Federation Proceedings

VOLUME 12 *September 1953* NUMBER 3

THE AMERICAN PHYSIOLOGICAL SOCIETY

Symposium on Communication in Biologic Organisms[1]

Chairman: E. J. BALDES

BIOLOGIC TRANSDUCERS[2]

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IN THIS PAPER I shall discuss certain features of the eye, the ear and, to a less extent, the non-auditory labyrinth, and Dr. Bullock will discuss other sensory systems. We will talk about the input to the central nervous system and Dr. Schmitt will discuss some aspects of the activity of the central nervous system from the point of view of communication. Dr. J. C. R. Licklider, who was originally scheduled to present the first paper, is a psychologist, and in substituting for him I deem it proper to introduce some psychological concepts. The ideas that I shall venture about the dimensions of vision and hearing have been developed in discussions with my psychological associate, Dr. Ira J. Hirsh (1).

My remarks divide into two parts: first, some generalities about the task of the exteroceptor sense organs in setting up patterns of nerve impulses that correspond to particular aspects of the external world and, second, certain features of the neurophysiological organization and behavior of these sense organs and their afferent neurons. I shall stress certain features that the three sense organs have in common and shall introduce only a few details concerning each one in order to clarify the common features. The most obvious statement about the eye, the ear and the non-auditory labyrinth is that each is specially sensitive to a particular form of energy: the eye to light, the ear to sound and the non-auditory labyrinth to the mechanical forces of gravity and acceleration. But we can also add that both the eye and the non-auditory labyrinth are so organized that they are sensitive to the direction in space (i.e., the azimuth) of these forms of energy.

The optical system of the eye forms a pattern of stimulation on the sensory surface of the retina that is a map of the external environment. The relative directions of the external sources of illumination with respect to the eye are here represented. The nervous system thus deals directly with an analog or model of one (two-dimensional) aspect of the external environment. The map changes as the eye (or the organism) moves relative to the environment or as external objects move relative to one another. The images of objects on the retina move about, but they do not, in general, suddenly come into existence or cease to exist except as one object passes in front of and obscures another.

If we become psychologists for a moment and consider the subjective 'output' of the visual sensory system, we recognize that the output is in terms of 'perceived objects' that can move in space but continue to exist. It is not my task to explain how this is accomplished, nor shall I pause to consider the development of the relation between visual objects and the corresponding tactile and kinesthetic objects. I may add here that throughout my discussion I am purposely omitting all discussion of the representation of the intensity of the stimulus, either in the analog process in the sense organs or in the subsequent neural activity. I will add also that the over-all sense of vision does give us some information from which we infer the distance from us of the external objects whose two-dimensional images fall on our retinas. The distance cues take the form of accommodation, convergence,

[1] Chicago, Illinois, April 7, 1953.

[2] This work was carried out under Contract N6onr-272 between the Office of Naval Research and Central Institute for the Deaf.

parallax, binocular disparity of images and so on. But here the third dimension in space is a sort of second-class citizen, in contrast to its direct representation in the non-auditory labyrinth.

The organization of the non-auditory labyrinth is in terms of three orthogonal axes in space. The sensory surface of this labyrinth is divided into pieces, and the pieces are associated with an elaborate three-dimensional apparatus that results in stimulation of one or another bit or bits of this surface when we are accelerated in linear motion in any of the three orthogonal directions or in rotation about any axis. The three dimensions of space are all directly represented, not only two of them as on the surface of the retina. In addition, one direction, up and down, is specially characterized by the constant acceleration of gravity. The 'object perceived' by this sense is the organism itself, oriented in a three-dimensional space, and accelerated or not accelerated relative to this space. Mathematically we might condense this proposition into a statement about the second derivative of space with respect to time.

In vision and in the sense of acceleration time is involved, of course, in that responses to stimulation begin and end. There is no further specialization in the direction of a special sensitivity to time except as time is intrinsically tied to space in the form of motion or of acceleration, i.e. the first and second derivatives of space with respect to time.

Hearing, on the other hand, is sensitive to only one spatial direction, namely azimuth in the horizontal plane, and even this sensitivity to a spatial dimension is indirect and requires binaural hearing or else scanning movements of the ears or head. Hearing is, however, directly and peculiarly sensitive to time. The sensory surface of the cochlea is not three-dimensional like the non-auditory labyrinth nor two-dimensional like the retina, but essentially one-dimensional. (If the transverse dimension of the organ of Corti has any special significance it is probably in the dimension of intensity.) The dominant dimension in the ear, along the organ of Corti from base to apex, is scaled in terms of frequency. High-frequency sounds stimulate only restricted areas in the basal turn of the cochlea, low-frequency sounds stimulate much greater lengths of the sensory surface and particularly the middle and apical segments. Now the dimension of frequency is events per unit of time. Position of stimulation in the sense organ is thus correlated with how fast something is happening. The thing that is happening in the external world in the case of a steady tone is a vibration or oscillation, usually of a solid object or of a partially confined volume of air. The ways in which an object vibrates or resonates when it receives a mechanical impact, or suddenly ruptures, or when air moves rapidly past it determine its pattern of frequencies of vibration. These patterns and the lengths of time that they persist give us information that allows us to recognize, if we have had normal previous auditory experience, what kind of an object (large or small) or what kind of material (hard, soft, or gaseous) is probably involved in producing the sound. Even without previous experience with the particular pattern we know that something is happening. The 'object perceived' is not the tangible or visible object but the event, namely its vibration.

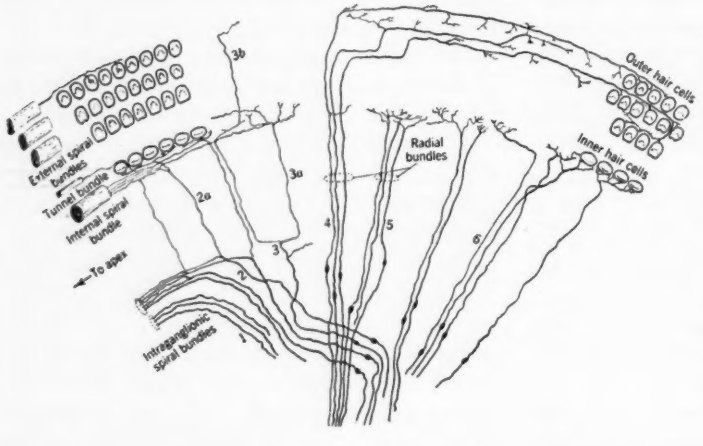

Fig. 1. INNERVATION of the chochlea. Diagram represents only a narrow sector and hair cells are indicated only in part. The following types of fibers are represented; intraganglionic spiral fibers, *1* and *2*; internal spiral fibers, *2a*, *3a*; external spiral fibers, *4*; internal radial fibers, *5*, *6*. A fourth class, the external radial fibers, is not illustrated. Based on Retzius, Solovocov and Lorente de Nó. (Reprinted with permission from WEVER, E. G., *Theory of Hearing*, New York: John Wiley and Sons, 1949.)

Sound waves that are rapid enough to be perceived as the pitch of a tone occur on a scale of psychological micro-time which the nervous system cannot resolve into the discrete events of individual sound waves. It is the rate of occurrence, not the individual event, that is represented by the locus of stimulation in the ear. But the ear also deals with a psychological macro-time, the time that we perceive not as the pitch of a sound but as the successiveness or the rhythm of discrete events. We know that the macro-time of successive events is handed on unchanged by the ear to the central nervous system in the form of corresponding volleys of nerve impulses. We cannot pause now to consider adequately the interesting area of overlap between the psychological and the physiological time scales. Actually we should probably recognize three orders of time: first, the macro-time of psychology in which successive events are perceived as separate and successive; last, a physiological micro-time in which rate of vibration is represented purely by the locus of stimulation of the sensory surface of the cochlea; and between them an overlap or meso-time in which volleys of nerve impulses correspond to individual sound waves and their frequency contributes to our sense of pitch. This meso-time is macro-time for peripheral neurophysiology but micro-time for psychology. It covers the frequency range from about 10 or 20 cycles per second to 1 or 2 kilocycles per second. Our interest lies in the overwhelming predominance of time as the dimension of hearing, and the importance of abrupt events, such as mechanical impacts of external objects, as the basis of many audible signals. These events in macro-time, the impacts, set up whole trains of events, namely vibrations, in meso- and micro-time. The model that exists on the sensory strip of the cochlea is a model of patterns of vibration, i.e. of rates of occurrence of the events in psychological micro-time. These patterns are handled in a manner analogous to the handling of directional, that is spatial, relations in the polar coordinates of vision. In each case an analog process is set up which is a spatial distribution of 'excitation' on a sensory surface. In more detail, I believe that this analog in the ear is a distribution of fluctuating electric current flowing from or through the sensory cells and exciting the nerve fibers that are attached to them, and the retina may very well operate in just the same way.

My main points so far are two. First, the eye, the non-auditory labyrinth and the ear sample the external world in different ways with respect to its three dimensions of space and one of time. (I hope that Dr. Schmitt can tell us something of how these three kinds of partial information, i.e. the visual world, the auditory world, and the inertial world, are synthesized in the central nervous system into a single spatial and temporal frame of reference in which objects exist, events occur and the organism moves.) Second, in each of the three sense organs there is a sensory surface or surfaces on which an elaborate sense organ builds up an analog or model or some aspect of the external world. This pattern is very different from the groups of nerve impulses that traverse the neural pathways of the brain. I want to suggest further that perhaps analog processes and models are not confined to the sense organs but may exist also in the nerve 'centers' where many neurons interact. The nervous system may be more than just a complicated network that operates entirely in terms of binary digits.

Now let us turn to some of the common features of the neurophysiological organization of the eye and the ear.

Even if there are analog processes or maps or models in the central nervous system as well as in the sense organs, the information is transmitted from the sense organs to the centers by the familiar all-or-none impulses. I shall venture a few generalizations about these impulses and their patterns as they leave the sense organs. Here we have some experimental facts, and they are probably more relevant to the remainder of this symposium than any speculations in which I could indulge as to the mechanisms by which mechanical or photochemical processes in the sensory cells might generate electric currents and thereby stimulate the nerve fibers that are attached to them (2–4).

I need hardly remind you that each of the great sensory systems contains thousands of individual neurons that serve as channels to conduct the all-or-none impulses. Each of these primary neurons innervates only a small part of the sensory surface of the sense organ, and the topological relationships of the sensory surface are preserved, in part at least, throughout the neural pathways to the sensory projection areas. It seems to be a general rule, however, that the relation between a primary sensory neuron and the sensory cells is not simply one-to-one. This first-order neuron is usually a simple bipolar cell, but it branches peripherally and each nerve fibre connects with more than one cell. Also each

sensory cell is innervated by more than one nerve fibre. Perhaps this arrangement represents, among other things, a margin of safety or redundance so that no one particular cell or neuron is indispensable for normal over-all function. This arrangement is particularly clear in the ear, where two systems of widely branching neurons and two other systems of more restricted neurons have been identified (5).

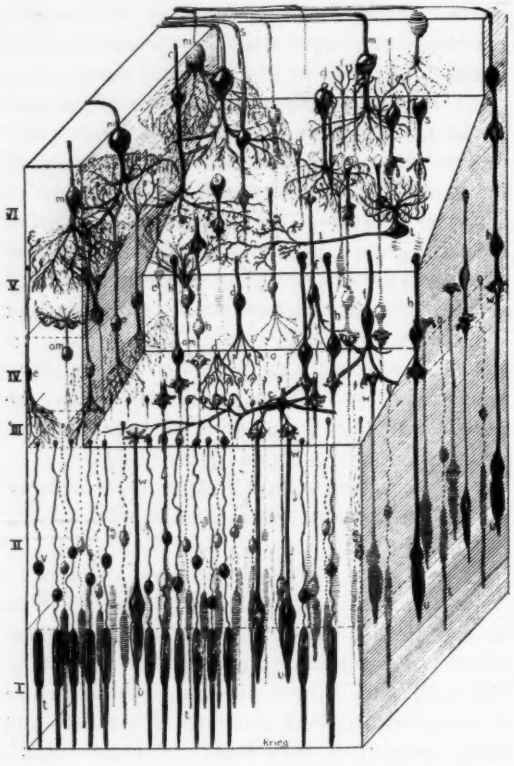

Fig. 2. STEREOGRAM of neurons in retina. The upper surface is towards the center of the eye, the lower surface borders on the pigmented layer. The upper layers have been partly trimmed away. *Layers*: I, rods and cones; II, rod and cone cells; III, outer fiber layer; IV, bipolar cells; V, inner fiber layer; VI, ganglion cells. *Cells*: (am) amacrine cells; (e) external horizontal cell; (d) bipolar cell of d-type; (e) bipolar cell of e-type; (f) bipolar cell of f-type; (h) bipolar cell of h-type; (l) internal horizontal cell; (m) large ganglion cell, (s) small ganglion cell; (t) receptive part of rod cell; (u) receptive part of cone cell; (v) central process of rod cell; (w) central process of cone cell. (Based on Cajal, Polyak.) (Reprinted with permission from KRIEG, W. J. S. *Functional Neuroanatomy.* Philadelphia: The Blakiston Company, 1942.)

The functional significance of the different anatomical classes of primary neurons in the ear is not clear. It is tempting to speculate that the wide branching of the spiral neurons may favor a great sensitivity by taking advantage of simultaneous excitatory activity in several receptor cells, while the more discrete innervation of the radial fibers is the basis of fine discrimination of the position of stimulation.

Our knowledge of the activity of the primary neurons of the eye and of the ear is very meager, largely because they are so closely associated with the sensory cells and the entire sense organ that it is difficult to reach them to study their individual activity and still maintain normal function in the sense organ. In the eye the sensory cells (rods and cones) and the bipolar cells, lie entirely within the retina and are overlaid by the ganglion cells. The latter are the largest as well as the most accessible, and all or nearly all of the studies of unitary neural activity of the vertebrate eye with micro-electrodes (6–12) apparently have sampled these higher-order cells almost entirely. In the ear Galambos and I (13) thought some 10 years ago that we had sampled the primary neurons in the auditory nerve of the cat, but we are now convinced that we were recording the activity of the cell bodies of second-order neurons of the cochlear nucleus. Only this morning was the first extensive report made, by my colleague, Dr. Tasaki (14), of the impulses recorded from certified first-order auditory neurons (in the guinea pig). But with two important exceptions, which I shall note in a moment, the relations found by Dr. Tasaki between the auditory stimulus and the discharge of impulses are very similar to the relations described by Galambos for the second-order neurons.

I shall mention three aspects of the activity of ganglion cells in which the eye, the ear and perhaps also the non-auditory labyrinth seem to be similar.

First, each nerve cell has not a receptive point but a receptive field. For one small area within the field (spatially on the retina and within a frequency range in the ear) the cell is most sensitive. In outlying regions, or for higher and lower frequencies, the threshold becomes progressively higher. In the eye, neural organization and interactions may be chiefly responsible for the gradient. In the ear, it is due in large part to the known physical behavior of the cochlea as a frequency analyzer, but even here we have evidence of a contribution from neural interaction. The receptive fields or 'response areas' of the second-order neurons are narrower, i.e. more 'sharply tuned,' than the very broad response areas found by Tasaki for the primary neurons.

Second, many neurons show a 'spontaneous' discharge of impulses at a relatively slow rate, unlike the familiar high-frequency injury discharges, that continue in the absence of or independent of any special auditory (or visual) stimulus. The primary auditory and labyrinthine neurons also show spontaneous discharges very frequently. Not all higher-order neurons are active in this way by any means, but spontaneous activity is very prominent in the dark-adapted eye and frequent in the ear, and we must consider the possibility that it has a physiological significance for the communication of information to the higher centers.

Finally, we encounter inhibition at the level of the second-order neurons. Either spontaneous activity or low-level evoked activity may be inhibited by other auditory (or visual) stimuli of appropriate intensity and frequency (or location). Here the primary and the higher-order neurons differ. Dr. Galambos (15) found that the discharge of about one-half of the spontaneously active second-order auditory neurons could be inhibited in this way. Dr. Tasaki found no instance of inhibition in the several dozen first-order auditory fibers that he studied. In first-order neurons from the otolith organs there is frequently a 'resting' discharge that is increased by rotation in one direction and decreased by rotation in the opposite direction (16–19). The latter effect might be classified as 'inhibition,' or it might be interpreted as due simply to the cessation of a steady stimulus. This kind of peripheral inhibition or suppression of spontaneous activity is an interesting feature of this and perhaps other sense organs.

The 'off' discharge that follows the cessation of illumination is a very prominent feature of the activity of the ganglion cells of the retina. The 'on' discharge and the 'off' discharge may both be modified by simultaneous illumination or change of illumination of other parts of the retina. No corresponding 'off-effects' have been found in the auditory system. This 'off' activity of the visual system obviously makes the eye more sensitive to change in illumination. As we have said, the eye is organized to deal with relatively steady states of illumination distributed in space and with movement of external objects which implies changes in these states of illumination. The ear seems more closely related to events in the external world and shows no off-effects and very little after-discharge.

Inhibition is clearly involved in many of these complicated interactions, particularly the modification or suppression of on-effects and off-effects. In the auditory system inhibition seems to be part at least of the mechanism by which the response areas of second-order neurons become sharper than those of first-order neurons. The complexities of both systems, including many features of each that I have not mentioned, have already disproved many early hypotheses as to sensory function. For example, a simple one-to-one mosaic hypothesis for the organization of the auditory or the visual system is not satisfactory. We must think of receptive fields, of spontaneous background activity, and of facilitation and inhibition at the very first synaptic level. Patterns of activity, both spatial and temporal, seem to be the stuff from which our hypotheses must be made.

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COMPARATIVE ASPECTS OF SOME BIOLOGICAL TRANSDUCERS

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SENSE ORGANS in lower animals offer a wealth of material for consideration from the point of view of transducer mechanisms and biological coding, both in respect of the variety of environmental changes detected and of the complexity of the chain of processes leading to a coded message in the sensory nerve. I will pass over, for the present purposes, many fruitful chapters such as enumeration of the diversity of adequate stimuli for which transducers have been specialized in animals, consideration of their wide useful range, variable amplification, non-linearity, multiplicity and nonidentity of channels with varying degrees of cross-talk and convergence between them and large and often involved time-dependence manifested in adaptation, post-excitatory depression, depression by strong stimuli with release rebound and other signs of altered properties. However, there remain many chapters of some interest and the following remarks are selected from those where the variable of diverse species gives some perspective on the evolution of transducer mechanisms.

Unit receptors usually detect more than one parameter of a stimulus. The first point I would like to make about animal sensory receptors as transducers is their imperfection. In spite of great evolution from generalized sense cells in lower forms which detect a variety of environmental changes to the specific receptors of higher forms which, as Dr. Davis has just emphasized, are differentiated into a number of particularized detectors within each of the organs like the eye, ear and nonauditory labyrinth, still many of them are very imperfect in the sense that the single receptor unit detects more than a single parameter of the environmental change. This places a burden upon the central nervous system to sort out and interpret identical signals from the unit as having different meaning, based upon different recent histories of signals from that unit or from other units.

I will mention two cases by way of illustration. And, with apologies to Dr. Davis, I will draw some illustrations from visual and acousticolateralis organs or their analogues, when comparative studies seem to make a point of general interest.

In the equilibrium sense organ of lobsters, a fairly typical statocyst, experiments in progress in our laboratory (1) have shown single units which are static position receptors, therefore having a certain 'spontaneous' frequency for each of certain positions, but which are also acceleration sensitive (fig. 1). This is reminiscent of the static and dynamic components of the sensory prepotential of muscle spindles discovered by Katz (2). In another organ currently under study, a temperature sense organ in the facial pit of certain snakes, illustrated in figures 2–5, (3, 4) the units are similarly dominated at first by sensitivity to the rate of change of temperature and then change over to a sensitivity to the magnitude first of the temperature change, then of the absolute temperature. For these separate sensitivities the difference limen, the resolution, the maximum impulse frequency, and the useful range of stimuli are widely different.

The same modality in different species uses different parameters. The second point I want to call attention to is that in different animals the same general function or modality, such as hearing, may sample quite differently or detect different aspects of the environmental change. An example may be taken from comparative work on sound perception, in order to underline the "sym" in this symposium. In insects, it appears that both tympanic organs and auditory hair sensilla are virtually pure displacement rather than pressure receptors; over a wide frequency range constant displacement produces constant response (5), with a threshold displacement of the distal process of the primary sensory neuron estimated to be 0.5 Angstrom units, possibly much less. One consequence of this in the tympanic organs, which like good displacement transducers are nearly undamped and inertialess is the pronounced directionality of each ear,

quite in contrast in each of these respects to our own ears which approximate pure pressure transducers. But, more important, it seems clear that crickets are quite indifferent to the parameter that means most to us, namely frequency, and instead are particularly sensitive to amplitude modulation frequency.

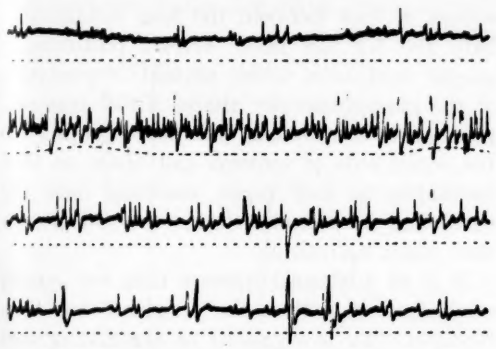

Fig. 1. NERVE IMPULSES in a bundle of a few dozen fibers in the right statocyst nerve of the spiny lobster, *Panulirus interruptus* (kindness of Mr. M. J. Cohen). There is continual activity of a certain level when the preparation is in the 0° ('normal' dorso-ventral) position (*first row*, top). This increases greatly during movement (*second row*; waves in the timing beam indicate rotation through 3°) and single unit analysis on records with only a few units firing shows this is participated in by the static receptors. In a new position 48° to the right, about the long axis of the animal, a new level of firing is maintained, here shown after 40 sec. (*third row*). Return to the 0° position after completing 360° of rotation (and some minutes later, *bottom row*) approximately restores the original level. Besides the static receptors thus qualitatively shown, various dynamic receptors can be shown in unit records. Time marks, 10 msec.

The same sensibility is used differently according to accessories. Just as in nonliving transducers, response to the same parameter of the same stimulus may be found in an organ whose secondary or accessory anatomy is markedly specialized in association with a special biological meaning or central interpretation of the information. Thus the warm receptors in the snake pit (figs. 2–5) appear to be basically similar to those in our skin but the secondary anatomy endows them with about a million times greater sensitivity in terms of caloric input, and hence the long infrared radiant flux from small objects at some distance and of low temperature differential is an adequate stimulus. Moreover, the anatomy is such as to permit a rather high accuracy of directionality. Something of the same kind can be said of the stress receptors which function as aerodynamic sense organs on the head of migratory locusts according to Weis-Fogh (6) and of the halteres of flies (7) whose stress receptors, acting in vibrating gyroscopes, detect inertial forces useful in maintaining equilibrium in flight. A number of sense organs employ accessory devices which have the effect of changing sensitivity; for example, by the reflex control of tonus in the contractile elements of the carotid sinus wall the useful range and the

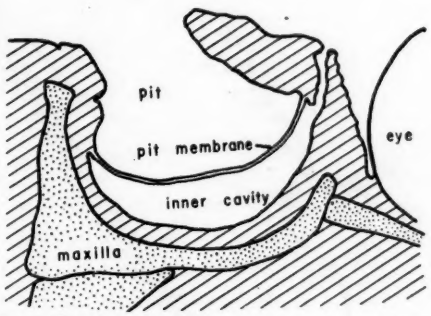

Fig. 2. DIAGRAMMATIC FRONTAL SECTION through the head of a pit viper (*Crotalus*) in the region of the facial pit. The pit membrane, about 15 microns thick but well vascularized and heavily innervated is the sense organ. Redrawn from Lynn (1931).

difference limen of the baroceptors is altered (8, 9).

Detectors for the same stimulus use different principles. Comparative studies on different animal groups have led to another general finding of broad significance, namely that different ways have been found by different animals of sampling the same environmental change, like microphones working on different principles. Returning to the lobster equilibrium sense organ, we are finding unit receptors with the same kind of response to the same kinds of stimuli, as is well known from the work of Lowenstein and others (10) in vertebrates. But here the whole receptor mechanism is radically different. Most important, the afferent neuron is believed to be itself the receptor, its terminal process being mechanically stressed because it penetrates into a hollow, movable chitinous hair. There is thus no hair cell, neuromast-equivalent or non-nervous sense cell which transduces the movement into a physiological event excitatory to nerve endings on it.

Similarly, hydrostatic pressure receptors in aquatic insects and vertebrates must work on quite different principles, although both must

begin with a compressible air space. The organs involved in vertebrates have not been satisfactorily studied physiologically but are certainly not very much like those recently discovered by Thorpe and Crisp (11), in certain permanently submerged bugs, in which sloping hairs in a special air space (differing from the respiratory air space in being compressible) are sensitive to uniform and to very small differential pressure changes which control orientation in both the planes of roll and of pitch.

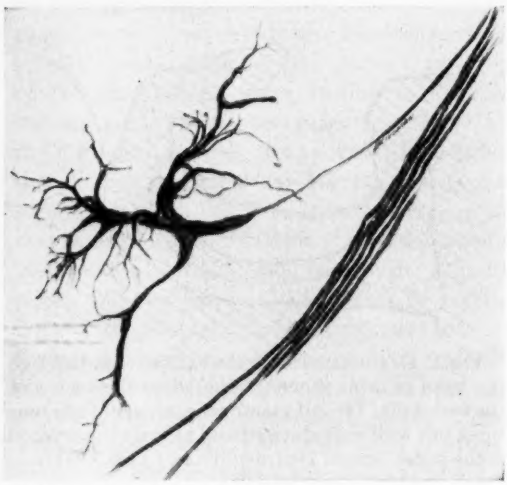

Fig. 3. Typical warm fiber nerve ending in pit membrane of the rattlesnake, *Crotalus viridis*. Reduced silver stain in a whole mount of the membrane (kindness of Dr. Wade Fox). The whole figure is 110 microns high. Fibers in the bundle are myelinated trigeminal nerve fibers, 2 microns in axis cylinder thickness at this point, 4–7 microns in total fiber diameter in the nerve trunk.

Familiar performance limits may not be 'theoretical' limits. There is another lesson that comparative physiology teaches which is impressively illustrated by a recent case in the domain of visual receptors, and that is that certain limits of performance of a sense organ, which are familiar to us from the human and the other commonly studied species, are not necessarily inherent in or close to the theoretical limits of the fundamental reactions or a measure of the latter, but may be exceeded by a large factor, presumably by superposition of a new device altering a time constant in a basically common mechanism. The remarkable case of this to which I refer is the so-called fast insect eye brought to light by Autrum of Würzburg (12). In certain flies, bees and dragonflies, the flicker fusion frequency is 180–250 per second, instead of 25–50 as in most insects studied. The electroretinogram shows a fast deflection at *on* and another at *off*, each one returning to the base line quickly, instead of the usual nearly monophasic pattern. The size of the ERG is virtually the same after long exposure at high intensity as at the beginning, that is, there is almost no light adaptation, or recovery from it occurs in the fraction of a second of dark between the long conditioning light and the test flash. Autrum postulates a special mechanism which prevents degradation of the photochemically altered visual pigment, presumably a substance like lumirhodopsin, to the equivalents of retinene and opsin, so that restoration is very rapid, requiring only the small step from lumirhodopsin to rhodopsin or their insect equivalents.

It is of additional interest that the central mechanism as well as the peripheral has been modified. This is suggested by the finding that the high flicker fusion frequency is demonstrable not only by recording electrically from the eye, but by behavior experiments (optomotor response). Moreover, by an ingenious method, Autrum has measured the temporal limits of the stroboscopic illusion, upper and lower, in various insects and finds they are displaced in the species with the fast eye far toward the high frequencies above those for the human and for other insects. Of course this must be a central phenomenon so the brain has readjusted its time scale along with the receptor.

Spontaneous receptor activity is common; steady static response and sensitivity. Dr. Davis has emphasized the importance of spontaneous activity in some receptor units. I would like to echo this emphasis and extend it by reminding you of the rather wide variety of sense organs in which it is characteristic and essential to normal function. These include the lateral line of fish—a delicate detector of water movement; the pressure receptors in the carotid sinus, gravity and acceleration receptors in lobster and crabs, as well as the vertebrate labyrinth, the dorsal abdominal stretch receptors in the crayfish (13, 14) and the temperature receptors in the three cases studied—the ampullae of Lorenzini in the skin of the ray (15), the cat's tongue (16) and skin (17, 18) and the snake facial pit. Now the importance of this continual activity is obvious for the signaling of equilibrium conditions, which as we now know is commonplace, that is in reception where no net energy flux is involved. Another result is the provision of an

off' signal, without requiring something especially activated by cessation of stimulation, but simply a decrease in the spontaneous or continual background. Another consequence is well illustrated by the temperature receptors, namely sensitivity to very small changes. It would be hard, perhaps, to imagine a nerve impulse being produced in a silent-until-stimulated neuron by a temperature change of 0.003° C, but this signal

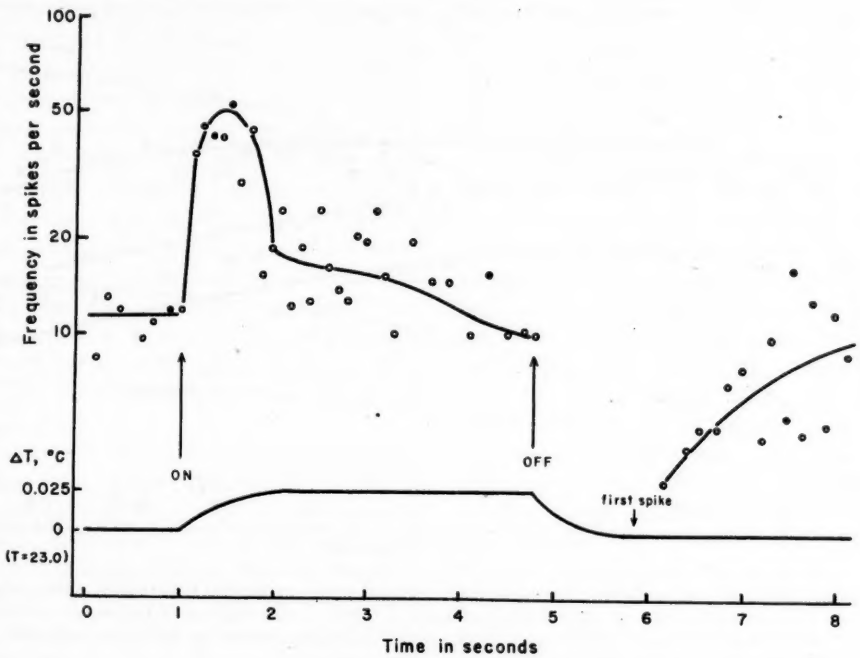

Fig. 4. Response in a two or three fiber preparation of the superficial branch of the supramaxillary nerve of the rattlesnake, *Crotalus*, to a sudden small increase in temperature of water flowing over the pit organ.

is quite adequate for a single temperature unit in the snake, increasing the frequency of firing from that at room temperature by at least 50%, providing the rate of change of temperature is adequate—0.003° C in 0.06 second in a certain experiment.

Actually this is not the limit of usefulness of the organ, but of the single units we have observed suitably. A 50% increase in frequency is ordinarily a large change; but here is a curious thing. We find these warm receptors, as Dodt and Zotterman (19, 20) do in the cat, in contrast to cold receptors, to be aperiodic or nonrhythmic until they are strongly stimulated. So far, no simple submultiple of the intervals between spikes has been found. This means that the background frequency is widely fluctuating and large or sustained changes in number of impulses per unit time are required in order to be sure of a response. This seems hardly to be adaptive, and invites speculation as to the meaning or origin of the nonrhythmic activity, which is a feature shared by several other spontaneously active sense organs—but not by all. Among several possible explanations, e.g. the interaction of random fluctuations of threshold and a slow recovery curve of excitability after each impulse, we might especially note that one which supposes the basic fluctuation to be rhythmic but to occur independently in each of several loci in the receptive arborizations of the first order sensory neuron. First this one and then that one will succeed in invading the main fiber. The anatomy of the endings, at least in the snake pit, seems to favor this and electrical evidence suggesting it has been described by Katz (2) in muscle spindles.

The nonrhythmic activity and consequent poor sensitivity to small short-term changes in frequency of impulses creates a special importance to spatial summation or the simultaneous stimulation of many receptors. Here is another central problem. Assuming the brain can integrate the small, individually unreliable changes in fre-

quency of many units to give a reliable total change, as we can by ear using the spikes of many units in a loudspeaker or with a simple R-C integrating circuit, an increase in useful sensitivity of at least several-fold could be realized. It would take only a factor of two or three to bring our figure of 0.003° C down to that which we calculate must be the temperature change due to radiation from a mouse at 50 cm in 100 msec.

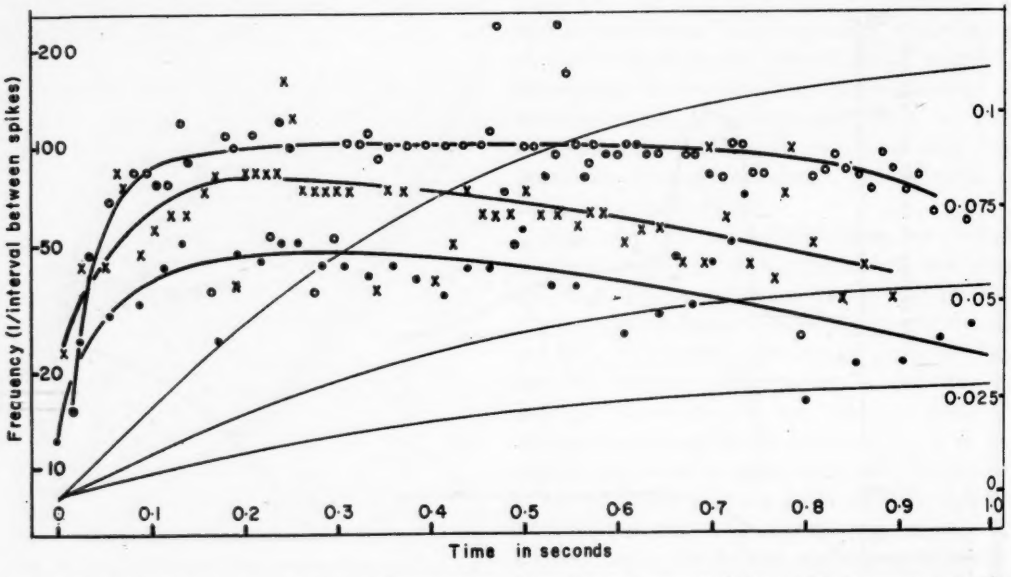

Fig. 5. Three different rates of rise of temperature plotted on an expanded time scale (same preparation as fig. 3). Temperature change on right hand ordinate. *Upper, middle* and *lower* response curves (circles, crosses and dots, respectively) correspond each to the stimulus curve in the same relative position. Note that definite response has occurred in less than 50 msec.

Very possibly the snake actually does better; this is a rather ordinary behavioral accomplishment (21). The human has a ratio of thresholds of single warm spot to large area (200 cm²) summated of about 1° C–0.003° C according to data from older literature (22, 23). Of course this may be partly due to the central nervous system ignoring even a fairly high frequency from a single unit.

Besides the sense organs already mentioned where spontaneous background activity is known to be essential to normal function, it is becoming apparent that random background discharge is a characteristic feature of others such as the eye (24) and the muscle spindle (2, 25).

Duplicate channels with different gain, time constant, and maximum output. There is one more topic in the area of interdependence of receptor and nervous system which I think is an example of our theme. This is, in brief, the generalization which has been proposed by Katsuki and coworkers (26), that in many sense organs which are supplied by nerve fibers of significantly varying diameters, there are some fibers, as it happens the thin fibers, that carry impulses from receptor elements of lower threshold to physiologic stimuli, of lower rate of adaptation and lower maximum frequency of discharge, than fibers from other receptor units. Further, the thin fiber-elements are more prone to continuous, so-called spontaneous firing and often, but not always, they are anatomically more ramified so that a larger number of sensory endings converge on one nerve fiber. The thin fibers, in short, supply the more sensitive and tonic, the thick fibers the more discriminating and phasic receptor elements.

To direct electrical stimulation with linearly rising currents, applied to the end organs, the thin fiber end organs have a lower threshold rate of rise and lower maximum frequency, just as for the physiologic mode of stimulation, although for steeply rising currents or brief shocks they have the higher threshold.

This correlation is to some degree familiar already, but it seems not to have been widely considered, pro or con, and many of the cases which offer suitable data for comparison have come to light only recently. For several sensory

modalities there appear to be no data for thin fiber responses. Thus for the cochlear and the vestibular nerves thin fibers are known histologically but their properties have not been compared with thick ones. The same is true for taste, olfaction and temperature.

But for an impressive list of sense organs—about 9 or 10—we can now make the comparison. The best known is perhaps the lateral line of the Japanese eel, studied by Katsuki, Yoshino and Chen (26)—thick fibers subserve receptors of higher threshold to physiologic stimuli and to slowly rising electric currents, with fast adaptation, high maximum frequency, less spontaneity and more restricted area of branching. Their higher threshold to slow rising current agrees with properties of the axon according to Katsuki and to Sato *et al.* (27, 28) who have re-examined the interpretation to the contrary in the extensive study on accommodation by Skoglund (29) and offer convincing evidence from single fibers (in agreement with Skoglund's figures) that small spikes characteristically appear first and large spikes later upon stimulation with slowly increasing current.

Two clearly marked classes of stretch receptors in the lung have been distinguished by Knowlton and Larrabee (30). They are mainly separated by a great difference in rate of adaptation and the slowly adapting ones have the low threshold. (In contrast to all other cases, however, these appear to be the thicker fibers.) Nociceptive fibers, as expected from clinical experience and recently reported by Maruhashi, Mizuguchi and Tasaki (31) from single fiber recording follow the general principle. So also do thicker tactile and thinner light pressure endings in frog skin and around cat's hairs studied by the same authors and around cat's whiskers as found by Katsuki (26). Proprioceptors from muscle and tendon did not until recently fit in and, of course, the relation is not an essential or necessary one. But Kobayashi, Oshima and Tasaki (32) have found both thin and thick tendon afferents, by single fiber isolation in nerve trunks; the thin fibers were from more tonic and lower frequency receptors. In the muscle, besides the well-known tonic spindle nerve fibers they found some phasic ones with higher adaptation rate and some smaller myelinated afferents with very slow adaptation.

Dr. Wiersma kindly permits me to refer to his findings on the stretch receptor recently discovered by Alexandrowicz (33) in the abdominal musculature of lobsters and crayfish and some of their allies. This remarkable organ consists of two single specialized muscle fibers, each with apparently a single primary sensory neuron of large size. The thinner one is non-adapting and lower threshold and has more extensive terminal ramifications.

In the retina rods behave like thin fiber-receptors and cones like thick, at least in respect to threshold, adaptation and convergence. In the cochlea, Lorente de Nó (34) has already shown that many outer hair cells are supplied by each nerve fiber while inner hair cells more nearly approach a one-to-one innervation. It would not be surprising, then, if it is found that the former receive mainly thinner nerve fibers and the latter thicker ones and that the physiologic properties are different. Although it seems clear that some biological transducers find it convenient to arrange duplicate detectors—one set for greater sensitivity, longer time constant and less vigorous output, the other for more discrimination both of intensity of stimulus above threshold and of rate of change of stimulus, this principle is not on any present evidence to be expected universally and exceptions may be anticipated. Thus Landgren (8) finds small fibers to be of higher threshold in the carotid sinus baroceptors. However, here it is possible that the two sets of fibers are not to be compared because (9) one—the large fibers—innervates receptors acting in parallel with the contractile elements of the sinus wall, the other supplies receptors either in series or in some other orientation. They obey the principle, however, in respect to maximum frequency of firing and adapted frequency at any given stimulus level producing maintained discharge.

It may be justified to draw one further tentative conclusion from these facts relating to the basic mechanism determining temporal pattern of sensory impulse discharge, at least as to its locus. Of the cases just cited, four are receptors with primary sense cells, i.e. true neurons, are supposedly the actual receptors—these are the pain endings, free nerve endings in the lung, the rods and cones and the crustacean stretch receptors. The others are supposedly dependent on true or secondary sense cells, like hair cells (lateral line), or are unknown in this respect. The suggestion of Katsuki, based partly on this agreement between organs employing and those lacking non-nervous sense cells and partly on close anatomical-physiological analysis of the

lateral line case, is that in all cases the characteristics of the discharge in the nerve fiber of the first order neuron are determined by the terminal part of that fiber, not by the nonnervous, secondary sense cell, when there is one. This suggestion is in contrast to widely held ideas of the role of the secondary sense cell, and should stand or fall independently of the suggestion on relative roles of thin and thick afferents. The properties of the receptor discharge to normal stimulation, according to this principle, match not only the diameter of the nerve fiber but all the properties that go with that—rheobase, threshold to brief shocks, threshold rate of rise of linearly rising currents, speed of conduction, spike height, accommodation and repetitiousness. This may be a close matching of secondary sense cell and the nerve fiber, but until we know better, perhaps it is the simpler assumption that the match is between a nerve fiber and its own terminals—which therefore would be determining the character of the discharge.

It is to be hoped in any case that these suggestions on general physiologic meaning from comparative data will stimulate closer analysis, especially in some of the higher and more studied organs. Certainly, the general physiology of excitation, at the molecular level—of the mechanism of transducing temperature, mechanical, chemical and radiant stimuli into impulses—is one of the most fruitful areas for future discovery.

Original work mentioned has been aided by grants from the University Research Committee, the National Science Foundation and the National Institutes of Health.

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AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

Symposium on Chemistry and Functions of Coenzyme A[1]

Chairman: FRITZ LIPMANN

INTRODUCTION: COENZYME A, PAST AND FUTURE

FRITZ LIPMANN

From the Biochemical Research Laboratory, Massachusetts General Hospital and the Department of Biological Chemistry, Harvard Medical School, Boston

DURING its short history, beginning in 1945, coenzyme A (CoA) already has made two rather outstanding contributions to biochemistry. The first contribution I would consider the bestowal of a metabolic function to pantothenic acid, the second, introducing a quite new and unexpected type of energy-rich bonding.

You may remember that the coenzyme was discovered as a component of a seemingly unimportant side reaction, the detoxication of aromatic amines. This acetylation system had been chosen for intensive study because it seemed so promising as an approach to the acetate activation problem. The implication of a new cofactor, CoA, was found and for this the sulfonamide acetylation system provided an ideal assay system. Then the discovery of pantothenate in CoA and its universal occurrence immediately suggested a metabolic function of prominence. More specifically, its implication in various acetylations and particularly in acetoacetate and citrate synthesis clearly suggested its function as an acetyl carrier. An even wider field of action was soon forecast by the discovery of its role in hippuric acid synthesis, covering not only acetyl, but rather acyl transfer in a much more general sense.

The burning question then arose as to how acyl transfer and acyl activation were to be explained in chemical terms. First thought turned to the pantothenic acid as the possible focus of activation. In our preoccupation with the vitamin, we were, it appears now, too oblivious to early observations on the presence of large amounts of sulfur, as disulfide, in all our CoA preparations. The preparation in which pantothenic acid was discovered, which, as we now know, was already 40% pure CoA, contained 2% sulfur. Furthermore, the need for a reducing agent in all CoA reactions should have caught our attention. The solution to the puzzle was presented by Lynen's brilliant work, which identified acetyl CoA as the acetyl thioester of CoA. This discovery of Lynen's introduced into the chemistry of intermediary metabolism an entirely new bond principle, which now already is permeating into other metabolic territories, overlapping in particular into the field of action of the most promising recent addition to the sulfur family, lipoic acid.

The great versatility of the mercaptoester link as an acyl transfer system is at the present time quite understandably overshadowing the pantothenic acid implications with this coenzyme. However, it might be kept in mind that nature certainly would not have placed this curious and, for the organism, rather valuable compound into the coenzyme without good reason. It remains a challenge to our chemical imagination to find out eventually why, in addition to the functional SH-group, pantothenic acid, obviously as a modifier, is built into the CoA molecule.

It appears more and more clearly how marvelously the sulfhydryl and disulfide bonds are suited for capturing and carrying acyl groups in activated form. But nevertheless, one gains the feeling that we should not overemphasize the sulfur bond. We rather should try to integrate it into the metabolic map where we find it lined up organically and in part overlapping with the energy-rich phosphate bond. The framework of the metabolic structure has to be

[1] Chicago, Illinois, April 7, 1953.

enlarged to accomodate the new bond type. Phosphate has lost its uniqueness as an energy carrier, but still, phosphate bond energy represents the most general energy currency. Phosphate bonding, it appears now, is pooling with other bond types. For the group transfer scheme, the new development means greater generality. The same stratification of bond energies is met with again in the series of acyl derivatives. Aceto ~ acetate, pyruvate (acetyl ~ formate) and the acyl ~ S.derivatives, correspond in energy content to the energy-rich phosphate compounds; the acyl-O-esters and acyl-N-peptides represent the 'energy-poor' partners, in parallel to ordinary phosphate esters. The details are changing, but the general picture becomes, if anything, consolidated; we are dealing with a well-defined pattern of group transfer which we now understand to include a variety of bonds, all clustering more or less closely around certain energy levels.

In addition to the now known ones, we have to expect still to turn up quite a few other bond types on the energy-rich level. Stadtman's acetyl ~ imidazole and the C ~ $\overset{+}{\underset{\|}{\text{N}}}$ in pyridine nucleotides (Kaplan and Colowick), in thiamine and similarly in folic acid (Wooley) come as most recent additions. They open promising new avenues for group exchange.

ENZYMATIC SYNTHESIS AND STRUCTURE OF CoA[1]

G. David Novelli[2]

From the Biochemical Research Laboratory, Massachusetts General Hospital and the Department of Biological Chemistry, Harvard Medical School, Boston

Ever since the discovery of coenzyme A (CoA) in 1945 by Dr. Lipmann (1), the isolation, purification, mechanism of action, and biosynthesis of CoA have been under investigation in his laboratory (2–5). Subsequently, other laboratories have undertaken the study of CoA-linked reactions and have made valuable contributions to our knowledge of the field (6–9). This afternoon's program will attest to the activity and contributions in this area. As a background for the discussion of the mechanism of CoA reaction, which will occupy the remainder of today's program, I would like to report on what we feel is the final structure of CoA and to present some of the evidence in support of this formulation. The final picture is the result of the combined efforts of a number of people who have participated in this project over the years. Besides those of us on today's program, I would like to acknowledge the participation of Dr. Nathan Kaplan, Dr. Morris Soodak, Dr. John Gregory, Mr. Frank Schmetz, Miss L. C. Tuttle, Miss Ruth Flynn and Mr. Edward Voelkel.

CoA was isolated and purified from a *Streptomyces fradiae* fermentation through a series of steps involving adsorption and elution from charcoal, reduction with Zn and HCl, and chromatography through a Duolite CS-100 resin column (10). We are indebted to Drs. Govier and Evans of the Upjohn Company for making available generous quantities of starting material for the isolation. By this procedure, CoA has been purified to well over 90% (3). I should point out that this isolation procedure has been improved by important contributions from Green's (11) laboratory and by Stadtman and Kornberg (12).

The molecular composition of one of these purified preparations is given in table 1. Assuming 1 mole of pantothenate per mole of CoA, the analysis indicates that CoA contains 1 M of adenine, 3 M of phosphate, of which ⅓ or 1 M is a phosphomonoester, and 1 M of sulfur. These data, then, give the essential composition of the coenzyme (3).

In order to reveal the linkages binding the constituent parts of the molecule, use was made of specific enzymatic degradations. In analyzing these enzymatically derived products, it soon became apparent that the test system for CoA assay was capable of resynthesizing the coenzyme from some of its degradation products (13). The assay system was that developed by Kaplan and Lipmann (14), namely, the CoA-dependent acetylation of sulfanilamide by acetate and ATP catalyzed by an extract of acetone-dried pigeon liver. Subsequently, other assay systems for CoA were developed which did not require the addition of ATP and therefore largely minimized the possibility of resynthesis from fragments. The most important of these assay systems has been the arsenolysis of acetyl phosphate catalyzed by the enzyme transacetylase from *Clostridium kluyveri* (15), a reaction discovered by Stadtman and Barker (16).

By means of such enzymatic degradations and resynthesis, together with the molecular composition shown in table 1, the structure of CoA has been established. This structure is shown in figure 1.

The coenzyme is a somewhat atypical dinucleotide, the usual mononucleotide being here replaced by phosphopantetheine. It consists of the vitamin, pantothenic acid, joined through a peptidic link to β-mercaptoethylamine. This latter peptide was first isolated from natural sources by Snell and his co-workers (17) as a growth factor for *Lactobacillus bulgaricus* and consequently named 'LBF' before its chemical nature was known. The peptide was subsequently synthesized by Snell *et al.* (18) and named 'pantetheine.'

[1] This work was supported by a research grant from the Office of Naval Research, contract NONR 606.

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At the other end, pantothenic acid is joined by a pyrophosphate bridge to an adenylic acid moiety. The adenylic acid has a phosphomonoester at a position corresponding to that of adenylic acid 'b', which has lately been identified as *position 3* on the ribose moiety (19). The position of this monoester was located by Kaplan and his co-workers (20) by the use of a nucleotidase from barley which is specific for the 'b' or *3 position* (21). The numbers and arrows on this figure indicate the points of enzymatic attack on the coenzyme.

a) Prostate phosphomonoesterase will attack at *position 1*, removing the phosphate group from ribose and giving rise to an inactive fragment which we have called 'dephospho-CoA'. This fragment is readily converted to CoA by crude pigeon liver extracts in the presence of ATP (22). The barley 'b' nucleotidase isolated by Kaplan and Schuster is about 20 times more active than the prostate enzyme in attacking this position.

TABLE 1. MOLECULAR COMPOSITION OF CoA

	CALCULATED %	FOUND %	RATIO
PANTOTHENIC ACID	26.6	26.8	1
ADENINE	17.6	17.0	1.05
RIBOSE	19.5	—	—
PHOSPHORUS	12.12	10.6	2.83
MONOESTER		3.6	0.96
SULFUR	4.18	4.13	1.07
MERCAPTOETHYLAMINE	9.9	—	—

b) A nucleotide pyrophosphatase from potato extracts or snake venom splits the pyrophosphate bridge at *position 2*, giving rise to diphosphoadenosine and phosphopantetheine (5). These products were isolated by paper chromatography and shown to be monophosphoesters. This constitutes the strongest evidence for the presence of a pyrophosphate bridge in CoA. This split of CoA at the pyrophosphate bridge is similar to the split of DPN and TPN earlier demonstrated by Kornberg (23). The fragment, phosphopantetheine, is likewise readily converted to CoA by crude pigeon liver extracts in the presence of ATP.

The structure shows the pyrophosphate bridge attached to the 4′ hydroxyl of pantothenic acid. The evidence from enzymatic degradation studies was insufficient to accurately place the position of attachment of the pyrophosphate bridge. It was only through studies on the biosynthesis of CoA from the various phosphopantetheines that this position was located (24).

We should like to acknowledge the cooperation of Dr. J. Baddiley of the Lister Institute in London in this phase of the work. He noted that mild alkaline hydrolysis of CoA gave rise to a phosphorylated pantothenate which migrated on paper like 4′phosphopantothenate which he had synthesized (25). We are indebted to him for supplying a large variety of synthetic compounds used in our biosynthetic studies. The details of this work will be discussed somewhat later.

c) If CoA is treated with both snake venom and prostate or with intestinal phosphatase alone, the pyrophosphate bridge (*position 2*) is cleaved and the resulting monoesters are dephosphorylated giving rise to 3 M of orthophosphate and 1 M each of adenosine and pantetheine. Pantetheine is very poorly converted to CoA by this crude pigeon liver enzyme.

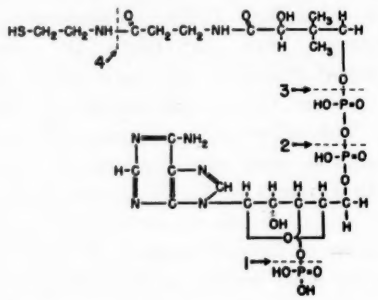

Fig. 1. STRUCTURAL FORMULA of coenzyme A.

d) Finally, CoA, dephospho-CoA, phosphopantetheine and pantetheine are all attacked at *position 4* by an enzyme from avian liver, hog liver, and kidney, splitting out β-mercaptoethylamine (5, 26). The product of this split is inactive and not convertible to CoA by extracts of pigeon liver. At the present moment, this appears to be the only irreversible enzymatic cleavage we have observed.

Biosynthesis. The fact that enzymatic attack at *position 1*, *2*, and *3* led to products which were converted to CoA by extracts of pigeon liver suggested that the reverse of these degradations might be the pathway for the biosynthesis of CoA. Of the 3 products formed, pantetheine, derived by treating CoA with intestinal phosphatase, was less readily converted to CoA. When synthetic pantetheine became available, two groups, Govier and Gibbons (27) at the Upjohn Company and King and Strong (28) at Wisconsin, reported a 10–20% conversion of

pantetheine to CoA by pigeon liver extracts. Both of these groups worked with aged extracts of pigeon liver. The aging procedure had been described by Kaplan and Lipmann (14) as an effective procedure to rid the extracts of indigenous CoA. Such a procedure makes it possible to study the resynthesis of fragments simultaneously with the assay of CoA, since these same extracts perform the CoA-dependent acetylation of sulfanilamide.

Last year, Dr. Levintow, working in Dr. Lipmann's laboratory, undertook an investigation of the first step in the conversion of pantetheine to CoA, which was suspected to be a phosphorylation of pantetheine (29). *a*) It was first found that, if CoA was removed from the pigeon liver extracts by treatment with Dowex rather than by aging, the extracts were much more active in converting pantetheine to CoA than the corresponding aged extracts. Apparently the aging procedure, in addition to destroying CoA, results in the inactivation of the enzyme. *b*) By fractionation of the extracts with protamine sulfate and adsorption and elution from calcium phosphate gel, the first step in the reaction, namely, the phosphorylation of pantetheine, was separated from the subsequent reactions. This enzyme was named 'pantetheine kinase'. The enzymes responsible for the subsequent conversion of phosphopantetheine to CoA remained together in the supernatant and this extract was used as an assay system for phosphopantetheine.

TABLE 2. PURIFICATION OF PANTETHEINE KINASE

Fraction	*N*, mg/ml	Actitivity CoA υ/ hr/mg N	Times Purified	Recovery %
Original extract	6.8	17.7		
Extract of second protamine ppt.	2.5	152	8.6	80
Eluate from calcium phosphage gel	1.7	284	16	50

Taken from Levintow and Novelli (29).

Table 2 summarizes the purification of the enzyme. The enzyme was purified some 16-fold and was capable of making the equivalent of 280 υ CoA/mg N/hr., or roughly 1 μM of phosphopantetheine. Because of the low yield of product and the poor yield of enzyme from a large number of pigeons, isolation of the product was not practical and it was, instead, identified by indirect methods, as summarized in table 3.

The test employed in this experiment is the ability of the product, phosphopantetheine, to be converted to CoA by the enzymes in the protamine-treated supernatant. It is evident that pantetheine by itself is inactive in this respect. CoA either before or after treatment with prostate phosphatase is still active. This illustrates the conversion of dephospho-CoA to CoA. Phosphopantetheine, derived from CoA by enzymatic degradation, is convertible to CoA only when it has not been treated with acid phosphatase. The inactivity after acid phosphatase treatment indicates that the product was in fact a phosphomonoester which is dephosphorylated to pantetheine by phosphatase action, and as such is no longer convertible to CoA. In the last line of table 3, the product of pantetheine kinase is seen to behave like the phosphopantetheine derived from CoA by enzymatic cleavage.

TABLE 3. COMPARISON OF ENZYMATICALLY SYNTHESIZED PHOSPHOPANTETHEINE WITH PHOSPHOPANTETHEINE DERIVED FROM CoA

Compound tested	Treatment with phosphomonoesterase[1]	
	Before	After
	CoA units	
Pantetheine	0	0
CoA	2.0	1.9
Phosphopantetheine[2]	2.0	0
Pantetheine kinase product	2.0	0.1

[1] Prostate phosphatase. [2] Derived from CoA by treatment with snake venom pyrophosphatase.
Taken from Levintow and Novelli (29).

While these experiments clearly indicated that the product of the reaction between ATP and pantetheine was a monophosphopantetheine they could not tell us the position of the phosphate group on the pantetheine moiety. However, having available this protamine-treated supernatant which converts phosphopantetheine to CoA, it became possible to test a variety of phosphorylated pantetheine derivatives for conversion to CoA by this enzyme system. Dr. Baddiley very kindly supplied us with the 2′, the 4′ and the cyclic phosphopantetheines which he had synthesized and when these compounds were tested with this enzyme system, only the 4′phosphopantetheine was converted to CoA. The results are shown in table 4. All compounds were tested at 0.1 μM levels. The data show that pantetheine, 2′phosphopantetheine, and cyclic phosphopantetheine are all inactive. A dl mixture of 4′phosphopantetheine is converted to CoA

to the extent of about 40% while the d (+) 4′phosphopantetheine is 82% converted to CoA. This activity is to be compared with the 85% conversion of phosphopantetheine derived from CoA by treatment with snake venom nucleotide pyrophosphatase and the 90% conversion of the pantetheine kinase product. Baddiley has compared our snake venom product with his synthetic 4′phosphopantetheine by paper chromatography and has shown that both compounds migrate similarly (30). These data then establish the

TABLE 4. ENZYMATIC CONVERSION OF VARIOUS PHOSPHOPANTETHEINES TO CoA BY PURIFIED PIGEON LIVER ENZYMES

	% converted to CoA
Pantetheine	0
2′phosphopantetheine	0
Cyclic phosphopantetheine	0
dl 4′phosphopantetheine	39
d(+) 4′phosphopantetheine	82
Pantetheine kinase product	90
Product of snake venom CoA pyrophosphatase	85

All compounds were tested at 0.1 μM levels with aged protamine supernatant. Pantetheine kinase product was prepared by preincubating pantetheine and ATP with purified enzyme. Snake venom product was prepared from CoA by treatment with CoA-pyrophosphatase.

From Levintow and Novelli (unpublished).

fact that pantetheine kinase catalyzes a reaction between pantetheine and ATP, yielding phosphopantetheine with the phosphate group attached to the primary hydroxyl group. The subsequent conversion of this product to CoA is catalyzed by enzymes in the supernatant. These ideas are summarized on figure 2.

The reaction we have just been discussing is *reaction 1*, namely, the phosphorylation of pantetheine. This reaction is catalyzed by pantetheine kinase, which is precipitated by protamine sulfate. The subsequent conversion of phosphopantetheine to CoA is catalyzed by enzymes remaining in the supernatant. These reactions can be visualized as proceeding as indicated in *steps 2* and *3*. *Step 2* was postulated by analogy with the similar reaction in the synthesis of DPN described by Kornberg (31) and visualizes a condensation with ATP with the elimination of inorganic pyrophosphate yielding dephospho-CoA. *Step 3* would then be another kinase reaction involving the phosphorylation of the ribose moiety at *position 3*.

More recently, Dr. Mahlon Hoagland has undertaken the investigation of the two enzymes in the supernatant in an attempt to separate them and to establish the validity of the reaction mechanism given in figure 2 (32).

Because of the observation by Hogeboom and Schneider (33) that DPN synthesis occurs in the nuclear fraction of liver homogenates, this seemed to be a possible point of departure for the isolation of the enzyme which condenses phosphopantetheine with ATP. For the sake of convenience, we call this the 'condensing enzyme' (labeled C in the subsequent tables). Pigeon liver homogenates were prepared and fractionated into mitochondrial, nuclear, micro-

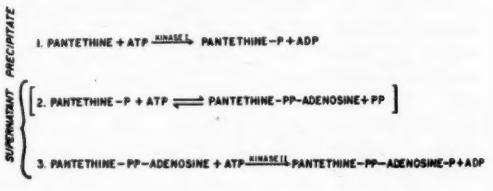
PRECIPITATE
SUPERNATANT
1. PANTETHINE + ATP → PANTETHINE–P + ADP
2. PANTETHINE–P + ATP ⇌ PANTETHINE–PP–ADENOSINE + PP
3. PANTETHINE–PP–ADENOSINE + ATP → PANTETHINE–PP–ADENOSINE–P + ADP

Fig. 2. ENZYMATIC SYNTHESIS of coenzyme A. Taken from Levintow and Novelli (29).

somal and supernatant fractions according to Hogeboom and his co-workers. Each fraction was tested for its ability to convert pantetheine phosphopantetheine and dephospho-CoA to CoA in the presence of Mg, cysteine and ATP. The microsomes were inert. Although the nuclear fraction appeared to have a little activity for the conversion of all substrates to CoA, it was most likely due to contamination with the supernatant fraction. In separate tests the nuclear fraction, especially after freezing or sonic disruption, proved to be inhibitory. The presence of a potent ATP-ase and CoA splitting activity may account for this nuclear inhibition. The mitochondria, though lacking pantetheine kinase, were quite active in converting either dephospho CoA or phosphopantetheine to CoA. Only the supernatant fraction contained all the enzymes necessary to convert pantetheine to CoA, and had the highest specific and total activity for the last two steps in CoA synthesis.

The liver supernatant containing all the enzymes for the synthesis of CoA from pantetheine at once afforded a greatly improved source of enzymes because the removal of particulate protein in the preparation of the supernatant fraction effected a 4-fold purification with complete recovery of activity, and because this procedure removed a potent nuclear inhibition.

Both rat and hog supernatants were quickly found to be excellent sources of enzyme. The easy availability of hog liver in large quantity made possible large scale preparation of the enzyme.

Using hog liver supernatant as a source of enzyme, a procedure has been worked out involving precipitation with protamine sulfate, ammonium sulfate fractionation, and adsorption of inactive protein with calcium phosphate gel, which gave approximately 100-fold purification of the last two steps in CoA synthesis. A summary of this purification is shown in figure 3.

PREPARATION	SPEC. ACTIVITY			RECOVERY %		
	K_I	C	K_{II}	K_I	C	K_{II}
Hog liver homogenate (after freezing)	1	1.5	2.0			
Hog liver supernatant		3	10		600	1000
Protamine supernatant		10	25			
38% $(NH_4)_2SO_4$ fraction	25	50	100	50	50	50
Ca PO4 gel 2.5 vol.	0	200	250		80	60
Ca PO4 gel 5.0 vol	0	150	70		20	
	C		K_{II}			
OVERALL PURIFICATION	100 x		125 x			
RECOVERY FROM SUPERNATANT	15%		30%			

Fig. 3. PURIFICATION OF CONDENSING ENZYME and dephospho-CoA kinase from hog liver supernatant. Taken from Novelli and Hoagland (32).

K_I is pantetheine kinase and it is removed by treatment with calcium phosphate gel. *C* and K_{II} refer to the condensing enzyme and dephospho CoA kinase. The use of 5 volumes of gel, although not giving as high a specific activity as the use of only 2 volumes, has the virtue of yielding preparations relatively more rich in C, completely free of ATP-ase, and greatly reduced in pyrophosphatase.

Since the enzyme preparations contained both the condensing enzyme and dephospho CoA kinase, any product formed would be converted to CoA. The CoA synthesized could be measured by means of the arsenolysis of acetyl phosphate catalyzed by the CoA-dependent phosphotransacetylase.

Dephospho CoA kinase (K_{II}) could be measured independently of condensing enzyme by using dephospho CoA as substrate. The experiment shown on table 5 demonstrates the almost quantitative conversion of dephospho CoA to CoA. The enzyme requires ATP and Mg^{++} ions, and the substrate must be in the reduced form in order for the enzyme to act. The enzyme is active over a broad pH range from 7.0 to 9.0. The reaction rate is linear with time up to about 80% conversion of the substrate.

Starting with phosphopantetheine and ATP, the condensing enzyme would make dephospho CoA. Since there is no specific method of measurement for dephospho CoA, the product of the condensation had to be converted to CoA and

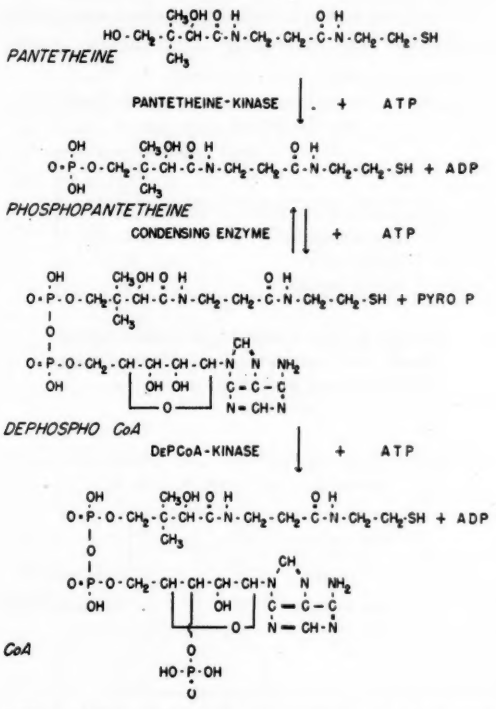

Fig. 4. BIOSYNTHESIS OF COENZYME A by hog liver enzymes. Taken from Novelli and Hoagland (32).

measured as such. If, however, the condensation proceeds as postulated earlier, that is, by splitting out inorganic pyrophosphate, then a more direct measure of the reaction would be the liberation of pyrophosphate.

The first clue that pyrophosphate might be involved in this reaction was the observation that the presence of added inorganic pyrophosphate inhibited completely the conversion of phosphopantetheine to CoA, while having no effect on the conversion of dephospho CoA to CoA. When the enzyme had been reduced in pyrophosphatase to the point where it was inactive at optimal mg concentration for CoA synthesis, it became possible to measure the formation of pyrophosphate in the reaction

mixture. The experiment illustrated in table 6 shows that when 5 μM of ATP are incubated with 2 μM of phosphopantetheine, a condensation occurs with the elimination of pyrophosphate in fairly good equivalence with the CoA synthesized. This experiment thus indicates that the condensation reaction in fact does proceed through the elimination of pyrophosphate from ATP and establishes the presence of a pyrophosphate bridge in CoA.

While kinase reactions in general are essentially irreversible due to a large drop in free energy

TABLE 5. SYNTHESIS OF CoA FROM DePCoA BY DePCoA-KINASE

	CoA μM
COMPLETE SYSTEM	0.18
MINUS ENZYME	0
MINUS ATP	0
MINUS DePCoA	0

ENZYME DePCoA-KINASE (K_{II}) 0.27 MG.; DePCoA 0.2 μM; ATP 1 μM; Mg^{++} 1 μM; CYSTEINE 10 μM; GLYCYL-GLYCINE BUFFER 40 μM, pH 7.4; 30' AT 37°

TABLE 6. CoA SYNTHESIS AND PYROPHOSPHATE APPEARANCE WITH K_{II} + C

SYSTEM	CoA μM	P-P μM	
ENZYME + ATP (5μM)	0	0	0.83mg protein
" + Pant-P (2μM)	0	0	Incubated 60',37°
" + ATP+Pant.-P	0.67	0.90	

between ATP and the monoester phosphate of the product, condensations between ATP and mononucleotides, with the splitting out of inorganic pyrophosphate, forming a new pyrophosphate link in the product, are theoretically reversible due to the fact that there is relatively no energy drop between the reactants and the products. Kornberg has already shown that the condensation of NMN and ATP splitting out pyrophosphate and forming DPN is readily reversible (31). We therefore attempted to reverse the condensation reaction by incubating dephospho CoA with pyrophosphate. The results of such an experiment are given in table 7.

Since both K_{II} and C are present in the enzyme mixture, the reactions shown at the bottom of table 7 are possible. By reversal of the condensation reaction, dephospho CoA is cleaved with inorganic pyrophosphate giving rise to pantetheine phosphate and ATP. Since kinase II is also present, the second reaction, namely, the utilization of the ATP formed in the first reaction to convert dephospho CoA to CoA will occur. The sum of the reactions would require 2 M of dephospho CoA to disappear for each mole of CoA formed. The table shows that no reaction occurs unless all reactants are present as in line 4. When 1.34 μM of dephospho-CoA are incubated with 2.0 μM of inorganic pyrophosphate, 0.5 μM of CoA are formed. The theoretical for complete reversibility would

TABLE 7. DISMUTATION REACTION WITH K_{II} + C

SYSTEM	CoA μM	
ENZYME + P-P (2.0 μM)	0	3.20 mg protein
ENZYME + de PCoA (1.34μM)	0	Incubated 65',37°
——— P-P + de PCoA	0	
ENZYME + P-P + de PCoA	0.5	

de P CoA + P-P $\rightleftharpoons$ Pant-P + ATP

de P CoA + ATP $\longrightarrow$ CoA + ADP

2 de P CoA + P-P $\longrightarrow$ CoA + Pant-P + ADP

have been 0.67. Therefore, this dismutation went approximately 85% of the way, illustrating the essential reversibility of the condensation reaction. CoA cannot replace dephospho CoA in this back reaction, and the enzyme is quite specific—NMN and ATP will not condense to DPN with this enzyme.

SUMMARY

By a combination of enzymatic degradations and resynthesis experiments, the structure and biosynthesis of CoA as been worked out. The biosynthesis of CoA from pantetheine in the supernatant of avian and mammalian liver proceeds in an orderly stepwise fashion as shown in figure 4. The first reaction is a phosphorylation of pantetheine on the 4'hydroxyl by ATP catalyzed by pantetheine kinase and yielding phosphopantetheine. Next phosphopantetheine is made to condense with ATP, splitting out inorganic pyrophosphate and forming a new pyrophosphate bridge between *position* 5 of adenylic acid and *position* 4 of phosphopantetheine and giving rise to dephospho CoA. Finally, dephospho CoA is again phosphorylated by ATP on the ribose moiety in a position corresponding to adenylic 'b' which is *position* 3'.

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DISCUSSION

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I THINK we can all congratulate Dr. Novelli and his colleagues on the beautiful work he has just presented to us. With the elucidation of the pathways of biosynthesis of CoA another chapter in the history of CoA is now closed. There is indeed nothing that the work of our laboratory could add to this problem, since we have not concerned ourselves with the nature of CoA but rather with its catalytic role in enzyme systems. Aiming at this latter objective, we have concentrated our efforts—in collaboration with Dr. F. M. Strong and his group—on making the coenzyme generally available for enzymatic studies (1).

Since Dr. Novelli has considered the enzymatic synthesis of CoA, it may not be out of place to make a few remarks on the enzymatic synthesis

TABLE 1. ENZYMATIC PREPARATION OF CoA DERIVATIVES

No.	Enzyme	Reactants	Product
1	Acetate activating enzyme	acetate / propionate + ATP + CoA	acetyl CoA / propionyl CoA
2	Fatty acid activating enzyme	fatty acid (C_4-C_{12}) + ATP + CoA	C_4-, C_5-, C_6-, C_7-, C_8-, C_{10}-, C_{12}-CoA, β-OH-C_4-, β-OH-C_6-, β-OH-C_8-CoA, $\Delta_{2,3}$-C_6-CoA
3	β-Hydroxyacyl CoA dehydrogenase	β-hydroxybutyryl CoA / β-hydroxyhexanoyl CoA / β-hydroxyoctanoyl CoA + DPN	acetoacetyl CoA / β-ketohexanoyl CoA / β-ketooctanoyl CoA
4	α-Ketoglutaric dehydrogenase	α-ketoglutarate + DPN + CoA	succinyl CoA

of CoA derivatives. The ways in which we have prepared a variety of these compounds are summarized in the table. Acetyl CoA and propionyl CoA may be obtained in good yield by 'activation' of the acids by ATP catalyzed by the acetate activating enzyme of animal tissues, *line 1* (2). The bacterial enzyme phosphotransacetylase has been used by Dr. Stadtman for preparation of acetyl CoA in excellent yield from acetyl phosphate (3).

The fatty acid activating enzyme, *line 2* (4), the activation mechanism of which also corresponds to that shown for the acetate activating enzyme, has proved in our laboratory to be a key enzyme for the synthesis of CoA derivatives. It reacts with a great variety of fatty acids from C_4 to C_{12} of straight, branched or substituted carbon chain as well as with unsaturated acids. The derivatives shown in the table are those which we have actually prepared with this enzyme. Their number is suggestive of the much greater number of those which may thus be prepared.

The fatty acid activating enzyme, which we obtain from beef liver mitochondria, has not only been a preparative but also a very valuable analytical tool. It has provided us with an assay for CoA which is independent of reaction rates and 'standard' CoA preparations—which are the basis for other assays of CoA. When an excess of ATP and a substrate of optimal chain length (C_7) are incubated with the enzyme, the conversion of CoA to the acyl derivative is essentially complete. The derivative reacts with NH_2OH to form the hydroxamic acid and the synthetic hydroxamic acid is used as a standard. Alternatively, disappearance of the free sulfhydryl of CoA may be measured. The assay is only applicable to relatively pure preparations of CoA. Glutathione does not interfere, since only acyl groups of short chain are transferred from CoA to glutathione.

The fatty acid activating enzyme has also been used for preparing the β-hydroxyacyl derivatives of CoA. These, in turn, can be converted to the corresponding β-ketoacyl derivatives in presence of a DPN requiring dehydrogenase, *line 3*. Dr. Lynen has demonstrated the formation of acetoacetyl CoA with the same dehydrogenase after preparing β-hydroxybutyryl CoA from acetyl CoA in two enzymatic steps (5). Dr. Stern and his colleagues (6) arrived at acetoacetyl CoA from succinyl CoA through a transferase reaction in which CoA bound succinate is replaced by acetoacetate (6, 7).

The oxidation of α-ketoglutarate by DPN in presence of α-ketoglutaric dehydrogenase has been used by Dr. Sanadi in our laboratory for the preparation of succinyl CoA, *line 4* (8). Although this compound can now be prepared chemically (9), the enzymatic method using α-ketoglutarate still has its virtue if label is to be introduced into succinyl CoA in an asymmetric fashion. Generally, we have preferred the enzymatic methods of preparation over the chemical ones, when labeled derivatives were to be prepared, because the commercially available labeled acids can be used directly as starting materials. With the preparation of a few enzymes a wide variety of acyl derivatives of CoA becomes thus easily accessible, the purity of which is of the same order as that of the chemically prepared derivatives.

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FUNCTIONAL GROUP OF COENZYME A AND ITS METABOLIC RELATIONS, ESPECIALLY IN THE FATTY ACID CYCLE

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THE CATALYTIC ACTION of every coenzyme is based on a stoichiometric chemical reaction between substrate and coenzyme. We call that part of the coenzyme which enters into reciprocal action with the substrate the 'reactive site' of the coenzyme, or, as Otto Warburg calls it, the "Wirkungsgruppe." Viewed in this way, the reactive site of coenzyme A[1] is the sulfhydryl group.

I should like to give you a short review of the way in which this discovery came about. The starting point of our experiments was the attempt to isolate active acetate from respiring yeast. While studying acetic acid degradation by yeast, a problem which was initiated twenty years ago by Wieland and Sonderhoff (1), I had come to the conclusion that this degradation occurs by way of the citric acid cycle, and that acetate is introduced into the cycle by the conversion into active acetate (2, 3). I may add that these ideas were proposed in 1941 when most biochemists supposed pyruvate to be the partner of oxaloacetate in citrate synthesis (4).

If the concept of acetate activation was correct, then yeast cells oxidizing acetate should be a rich source of active acetate. Three years ago, when Ernestine Reichert and myself (5, 6) started to isolate this substance from yeast, the brilliant contributions of Fritz Lipmann and his school (7), together with the later work of Earl Stadtman (8) and Severo Ochoa (9) suggested that active acetate is an acetylated derivative of coenzyme A. But the proof was still lacking.

Our test for the detection of active acetate was based on the enzymatic reaction with sulfanilamide. As shown in figure 1, in the presence of a fraction of pigeon liver, the acetylation of sulfanilamide increases with increasing additions of active acetate.

Using this procedure for the quantitative assay of active acetate, the purification of this substance became possible. Chemical investigations of crude preparations, as well as later preparations, which were about 40% pure, showed that the active substance is identical with S-acetyl-coenzyme A (5, 6). I have not the time to give an account of our proof of structure, which was substantiated in considerable detail. However, there were two observations especially important for the progress of our work which I should like to mention briefly.

One is concerned with the kinetics of the color development in the nitroprusside reaction (10). Figure 3 shows that the color development reaches a maximum in 2 to 3 minutes and then rapidly fades. Calculations of acetyl-CoA taken from the point of maximum color development are in excellent agreement with values obtained by sulfanilamide acetylation. Figure 4 shows a comparison of different preparations of acetyl-CoA assayed by the two methods.

The second observation relates to the absorption spectrum of relatively pure preparations of acetyl-CoA before and after exposure to alkali. We had learned from separate experiments that the ability to acetylate sulfanilamide was lost on treatment with alkali (6). This is associated with a change in absorption in the short ultraviolet as shown in figure 5. The decrease in absorption caused by alkali is plotted below as the difference spectrum, which has a maximum at 233 mμ and is due to the acetyl mercaptan group. Absorption of other thioesters in this region had been previously observed by Sjoeberg (11). Stadtman has also made this observation independently and applied it in a beautiful manner to the estimation of acetyl-CoA (12).

These results confirmed our ideas that acetyl-CoA is actually an acyl mercaptan and that the

[1] The following abbreviations are used in this paper: coenzyme A (reduced), CoA, CoA-SH or CoA̅-SH; S-acyl coenzyme A derivatives, S-Acyl CoA, acyl-S-CoA, acyl-S-CoA̅, or simply acyl CoA; oxidized and reduced diphosphopyridine nucleotide, DPN^+ and DPNH; reduced triphosphopyridine nucleotide, TPNH.

extraordinary susceptibility to alkali is due to the splitting of the thioester bond.

Turning now to the metabolic function of the sulfur bond, I mention first one very characteristic property of the sulfur bonded acyl group. From a chemical point of view it belongs to the acid anhydrides, for we must consider that thio alcohols, in contrast to normal alcohols, have the properties of an acid. It is in these terms that

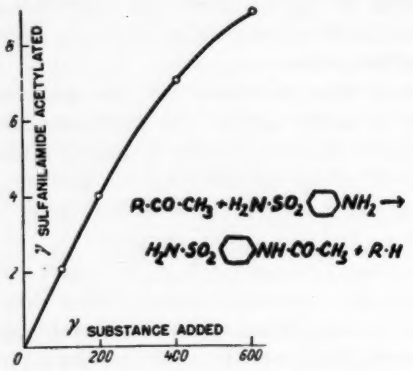


Fig. 1. ENZYMATIC TEST for 'active acetate' (6)

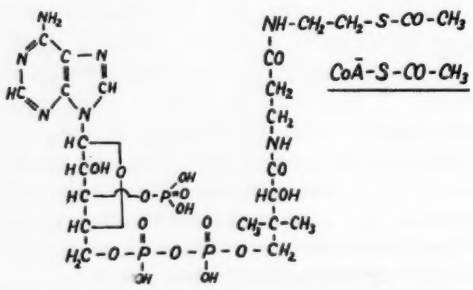


Fig. 2. 'ACTIVE ACETATE' = acetyl-CoA

the reactivity of acetyl-CoA as an agent for the acetylation of amino groups or hydroxyl groups, such as sulfanilamide or choline, is to be understood. We have to remember that the preparative chemist carries out acetylation of such groups with acid anhydrides such as acetyl chloride or acetic anhydride.

The peculiarity of the acyl mercaptan bond is the fact that in spite of the high energy of hydrolysis, it is completely stable in aqueous solution at neutral pH and room temperature, a property which permits the functioning of these compounds in cellular metabolism. Under physiological conditions the energy of the bond is realized only in the presence of specific proteins.

The attachment of the acetyl group at the sulfur of the coenzyme creates also a second site of reactivity. This is the labilization of the methyl hydrogen, which becomes apparent as an acidification. For technical reasons, the acidifying effect of the sulfur cannot be measured in derivatives of acetic acid, but can be clearly seen

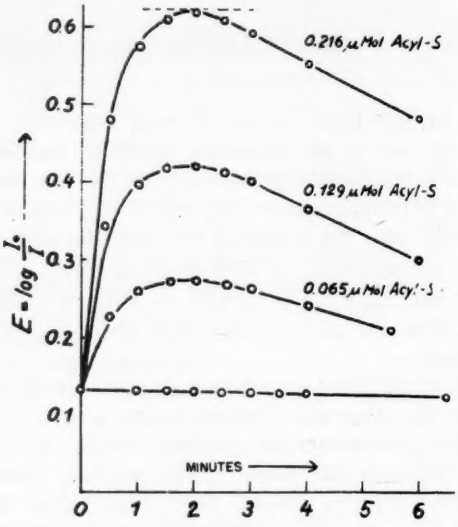


Fig. 3. KINETICS of color development in nitroprusside reaction, λ, 546 mμ (10).

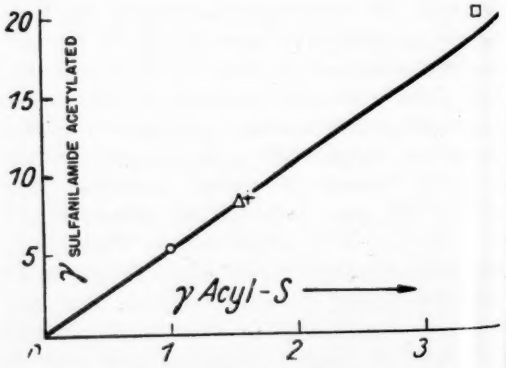


Fig. 4. IDENTITY of acetyl donor with acyl mercaptan (6). Preparations of varying purity from yeast Kochsaft: ○ = decomposed barium salt; + = charcoal eluate; △, □ = acetone precipitates.

in derivatives of acetoacetic acid. The results of electrometric titrations on acetoacetic acid, normal acetoacetic ethylester and on acetoacetylthioethanolamine are shown in figure 6. In the last compound the keto acid is bound to sulfur.

From the pK′ values of the dissociation it is seen that the acidity of the methylene group increases as one goes from the free acid to the ester and finally to the thio derivative. Since the

acidifying effect of the terminal acetyl group is the same in all three compounds, the difference in the pK′ values must therefore originate in the diversity of the second substituent, whether carboxyl, ester or acyl mercaptan group.

We thus have the significant demonstration that the α hydrogen atoms of an acid gain in acidity through binding of the carboxyl carbon to

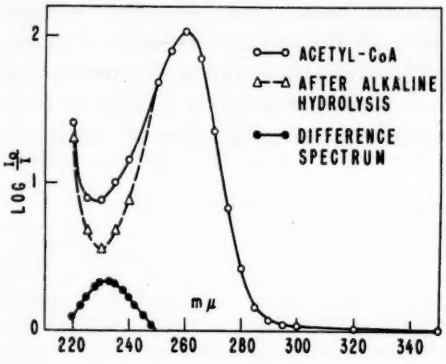


Fig. 5. Absorption spectrum of acetyl-CoA before and after alkaline hydrolysis. 1.56 mg acetyl-CoA (purity 0.40) in 10 ml M/20 phosphate, pH 7.4; light path = 1 cm. For hydrolysis the acetyl-CoA was incubated with M/4 KOH for 10 min. at 20°. Before absorption measurement this solution was neutralized with HCl.

$CH_3—CO—CH_2—CO—R \rightleftharpoons$
$[CH_3—CO—\overset{\cdot\cdot}{C}H—CO—R]^- + H^+$

$CH_3—CO—CH_2—COO^-$	pk′ = 12.70
$CH_3—CO—CH_2—CO—OC_2H_5$	pk′ = 10.70
$CH_3—CO—CH_2—CO—S—CH_2—CH_2—NH—COCH_3$	pk′ = 8.50

Fig. 6. Enol-dissociation of free and bound acetoacetic acid (13, 14).

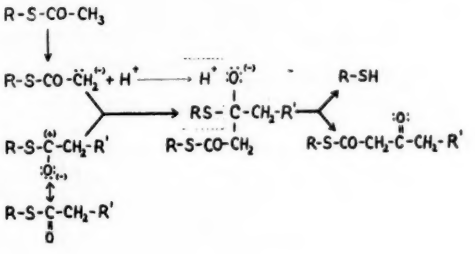
sulfur. This must also be true for acetic acid as well as the keto acids. I believe that this is the reason for the tail activation of acetyl-CoA so apparent in the synthesis of citric acid and the β-keto acids.

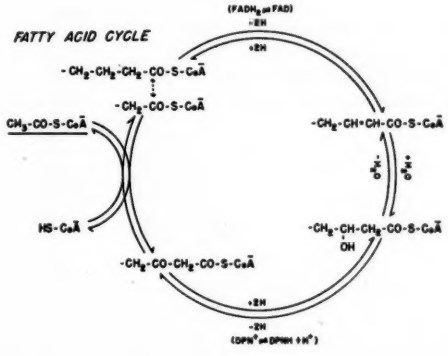
With this observation as a basis, we can visualize the chemical mechanism of the biological synthesis of acetoacetic acid, starting from acetyl-CoA.

Without going into details, this scheme shows how the primary condensation product is formed and then split into free CoA and acetoacetyl CoA. In the reverse reaction, the addition of the thio alcohol to acetoacetyl CoA forms the intermediate, which is then split into 2 molecules of acetyl-CoA. This process, the reverse of condensation, may be called thiolysis.

This condensation or thiolysis is the key reaction of the fatty acid cycle, the process by which long-chain fatty acids are built up or degraded by β-oxidation.

The term 'fatty acid cycle' is not quite correct. Each cycling of this process counter-clockwise elongates the carbon chain of the fatty acid-CoA derivative by 2 carbon atoms, using 1 acetyl-CoA



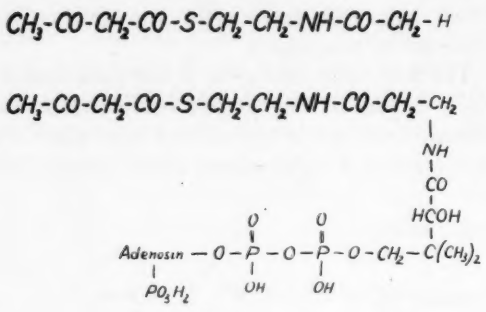


and 4 hydrogen atoms. In this direction the process might be better visualized as an expanding helix. For degradation of the fatty acids, the cycle proceeds in the clockwise direction in the form of a helix with decreasing radius.

The formulation of this cyclic process (5, 6, 15–17) was a logical consequence, once the chemical structure of acetyl-CoA became clear, because at this time a considerable amount of valuable experimental material on fatty acid metabolism was already in the literature.

It is the experience of modern biochemistry, which we owe to the pioneer work of Otto Warburg (18), that the study of enzymatic reactions as well as enzyme characterization, is greatly facilitated if the reactions can be followed by an optical test. When we began, after the acetyl-CoA work, to investigate the enzymatic steps of the fatty acid cycle, we tried first to

find optical tests for the individual reactions. Two types of CoA derivatives participate in the cyclic process. They possess quite characteristic



acetyl-thioethanolamine in water is shown in figure 7. A strong absorption appears in alkaline solution with a maximum at 303 mμ. For technical reasons we followed the changes at 313 mμ. This absorption band is to be attributed to the formation of an enolate ion and depends, therefore, as figure 8 shows, on the pH.

The increase in absorption coincides with the dissociation. The pK is 8.5, the value which was mentioned before (fig. 6). The absorption spectrum of the crotonyl derivative is shown in figure 9 and also the spectrum of free crotonic acid. It is evident that with the binding of the unsaturated acid to sulfur, there is a shift in the

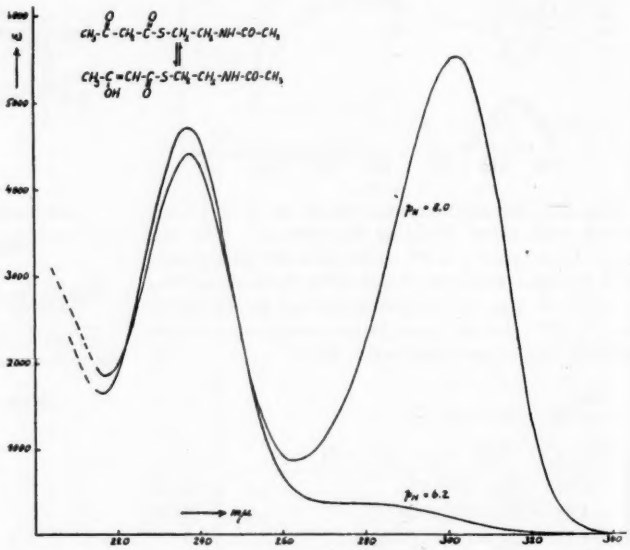


Fig. 7. ABSORPTION SPECTRUM of S-acetoacetyl-N-acetyl-thioethanolamine in acid and alkaline solution (14).

absorption bands, and their appearance and disappearance can be followed spectrophotometrically. These compounds are the CoA derivatives of the β-keto acids and of the unsaturated acids. Their absorption bands were discovered in my laboratory by Luise Wessely (14) and Werner Seubert (19). Their experimental approach to this problem was the synthesis of model compounds where coenzyme A was substituted by N-acetyl-thioethanolamine. For this purpose S-acetoacetyl-N-acetyl-thioethanolamine and S-crotonyl-N-acetyl-thioethanclamine were prepared. These compounds are structurally very close to the natural CoA derivatives. This is shown below for the two acetoacetyl compounds. The crotonyl derivatives have the analogous structure.

The absorption spectrum of S-acetoacetyl-N-

absorption of the double bond to longer wave length. Free crotonic acid has its maximum at 204 mμ (not shown in the figure) and the thioester at 224 mμ. At the same time a new band appears at 263 mμ, which may possibly be due to the S-CO-group. It seems to us that the shift of the peak to longer wave lengths is the reflection of the resonance between the double bond and the CO-group, a fact which is of great importance for the chemical reactivity of the double bond.

The optical behavior of the natural substrates, acetoacetyl CoA (15, 20, 21) and crotonyl CoA (22, 23) is essentially the same. The spectrum of acetoacetyl CoA is shown in figure 10. The only difference is that coenzyme A, in contrast to N-acetyl thioethanolamine, absorbs in the 260 mμ region because of the adenine group. This does not interfere with the enol band of acetoacetyl

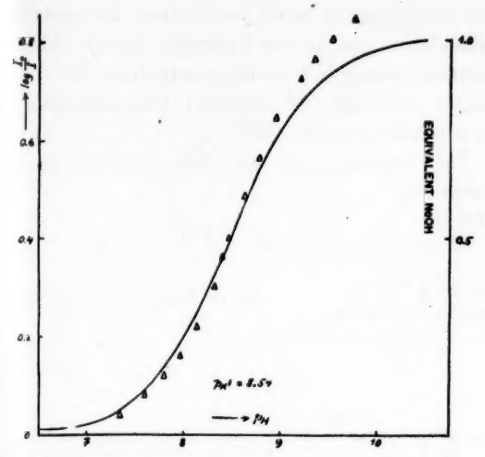


Fig. 8. TITRATION CURVE and pH-dependence of absorption at 313 mμ of S-acetoacetyl-N-acetyl-thioethanolamine (14). Solid line represents extinction of 5×10^{-5} M acetoacetyl derivative; light path 1 cm. Triangles (△) are points of titration curve. The deviation from the absorption at higher pH results from the instability of the thioester, even at 0°, because of the longer time necessary for the titration.

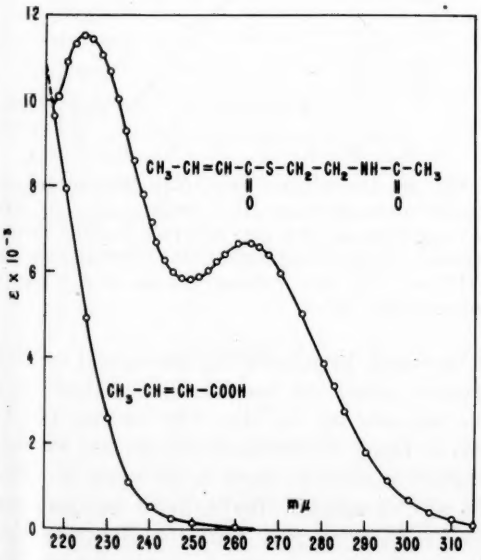


Fig. 9. ABSORPTION SPECTRUM of S-crotonyl-N-acetyl-thioethanolamine and crotonic acid (19).

thioesters but it does coincide with the double bond of crotonyl thioesters. Luckily the extinction coefficient in the latter case is so high that exact measurements of crotonyl CoA are also possible.

In contrast to this β-hydroxyacyl and saturated acyl CoA compounds have only the acyl mercaptan band at 233 mμ in addition to the adenine

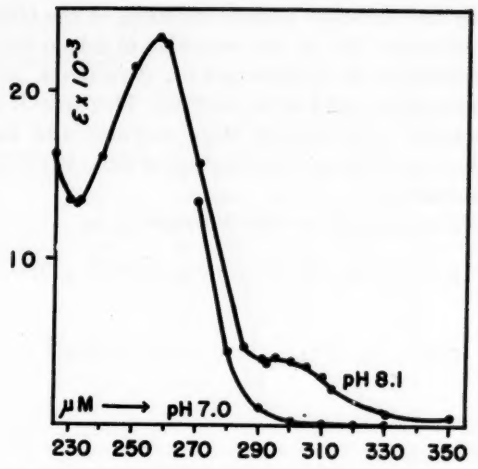


Fig. 10. ABSORPTION SPECTRUM of acetoacetyl CoA (cf. fig. 7). The preparation of acetoacetyl CoA, prepared by interaction of CoA with diketene (21), was 45% pure. The high absorption at 260 mμ is due to adenine impurities.

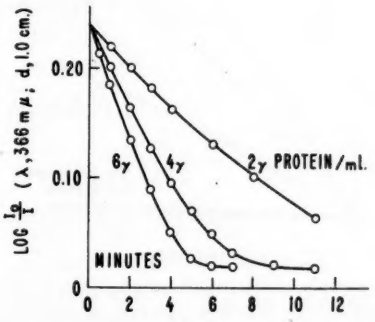


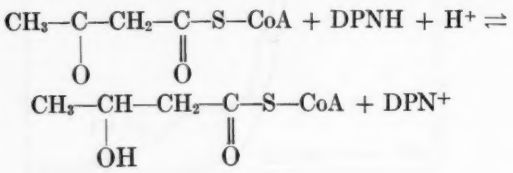
Fig. 11. β-KETO REDUCTASE TEST (15). Pyrophosphate buffer pH 7.4, 50 μM; ethylenediamine tetra-acetate, 2 μM; S-acetoacetyl-N-acetyl-thioethanolamine, 5 μM; DPNH, 0.13 μM. Volume, 2.0 ml; d = 1 cm.; temperature, 25°.

band. It is therefore possible in enzymatic experiments to follow the appearance and disappearance of keto acyl CoA compounds or dehydro-acyl CoA compounds if β-hydroxy or acyl CoA compounds are the precursors or the products of the reactions. A little later I shall come back to these tests, on which investigations in this field are fundamentally based.

It turned out that our model compounds were very useful in another respect. My collaborators Otto Wieland and Werner Seubert found that the more simply constituted thioethanolamine derivatives can substitute for the natural derivatives of CoA in the enzymatically catalyzed steps. As far as our experience extends, this is true for

the two hydrogen transfer reactions of the fatty acid cycle, that is, the reduction of β-keto CoA derivatives to β-hydroxy CoA derivatives and the reduction of the double bond. Therefore, it is possible to investigate these enzymes with the cheap model compounds instead of the costly CoA derivatives.

The equation for the first reaction is:



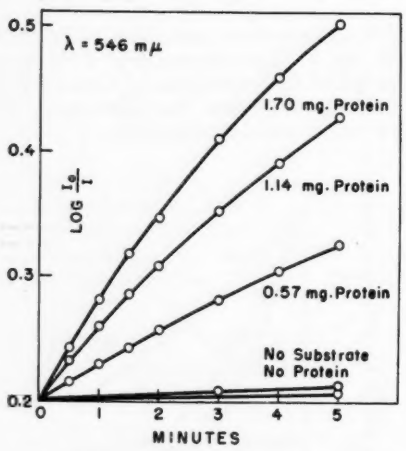
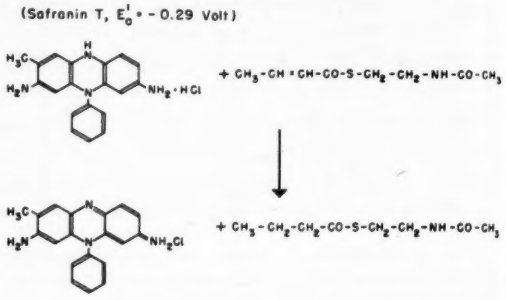


Fig. 12. ETHYLENE REDUCTASE TEST (19). Phosphate buffer pH 7.1, 138 μM; S-crotonyl-N-acetylthioethanolamine 2.6 μM; leuco safranine T 0.5 μM. Volume 2.1 ml; d = 0.5 cm; temperature 17°.

We call the enzyme which catalyzes this reaction 'β-keto reductase' (15). It not only handles the derivatives of CoA but also of N-acetylthioethanolamine. But the binding to sulfur alone is not sufficient because acetoacetyl glutathione shows no activity with the enzyme in our optical test (21). The test with S-acetoacetyl-N-acetylthioethanolamine and DPNH can be seen in figure 11. We follow the disappearance of DPNH.

Turning now to the second reduction step, the addition of hydrogen to the double bond, I may recall the fact that there was a resonating effect between the double bond and the S-CO-group. The result of this effect may be that thioesters of crotonic acid or longer α, β-unsaturated acids behave like α, β-unsaturated ketones toward reducing agents. The behavior of such ketones was demonstrated by Fischer (24) with his technique of using leuco dyes, for instance, leuco safranine as the hydrogen donor. Werner Seubert succeeded in demonstrating the enzymatic reduction of crotonyl thioethanolamine in a similar manner (19).

The equation of our test reaction is the following:



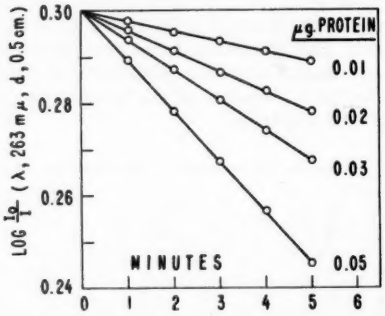


Fig. 13. CROTONASE TEST (23). Tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.5, 100 μM; egg albumin, 0.1 mg; ethylenediamine tetraacetate, 1.5 μM; S-crotonyl CoA, ~0.5 μM; 2.0 μM AMP in blank cell. Volume, 1.5 ml; d = 0.5 cm; temperature, 25°.

It can easily be followed by the optical method because during the reaction the colorless leuco dye becomes the red dye. The enzyme test is seen in figure 12. Without enzyme and without crotonyl derivative, there is no reaction. With the two combined, the activity increases as more enzyme is added. With the aid of this test Werner Seubert was able to purify the enzyme from sheep liver with an 80-fold increase over the initial activity. He accumulated strong evidence that it contains flavin-adenine-dinucleotide as prosthetic group (19). In this respect, 'ethylene reductase,' the name we propose for this enzyme, resembles the well known fumarate reductase. The function of the leuco dye is therefore to reduce flavin to hydroflavin, which then transfers hydrogen to the double bond. DPNH or TPNH are not able to replace the leuco dye.

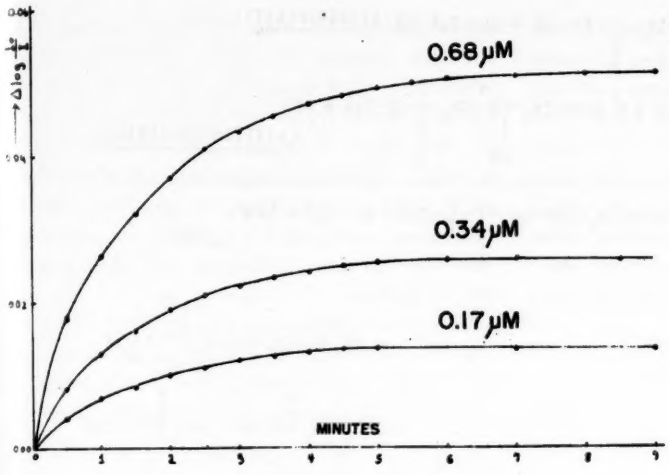
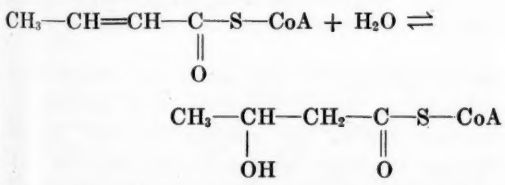


Fig. 14. SYNTHESIS of acetoacetyl CoA from acetyl-CoA with β-ketothiolase (14, 21). Pyrophosphate buffer, pH 9.0, 300 μM; ethylene diamine tetraacetate, 2.0 μM; β-keto thiolase preparation, containing 5 mg protein; acetyl-CoA, amounts given in the figure. Volume, 1.57 ml; λ, 313 mμ; d = 1 cm; temperature, 20°.

I turn now to the other enzymes of the fatty acid cycle. One is concerned with the addition or removal of water from the double bond, and the other with the condensation or thiolysis. The principle of the test reactions by which the activity of these enzymes may be demonstrated has already been mentioned. They are based on the absorption band of crotonyl CoA at 263 mμ and the enol band of acetoacetyl CoA at 303 mμ.

The test for crotonase as Raw and Stern (23) use it in the laboratory of Ochoa is seen in the figure 13.

Here crotonyl CoA is transformed to β-hydroxybutyryl CoA and therefore absorption at 263 mμ decreases:



Now turning to the condensation or thiolysis step of the fatty acid cycle, this interesting reaction can easily be followed by making use of the absorption band of acetoacetyl CoA at 303 mμ.

Figure 14 shows an experiment in which acetyl-CoA has been incubated with a purified enzyme preparation from sheep liver at pH 9. At this high pH, the synthesis of acetoacetyl CoA can be observed directly in the enolate form (14). However, the reaction soon comes to a stop because the equilibrium is far to the side of acetyl-CoA, that is to the side of thiolysis.

Following the reaction in this direction is not a practical method for measuring enzyme activity,

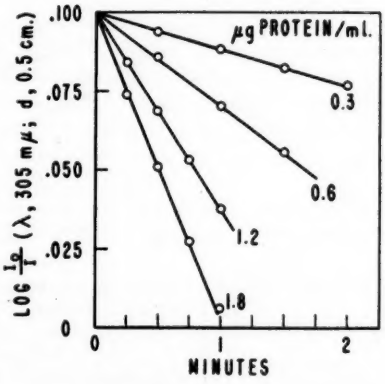
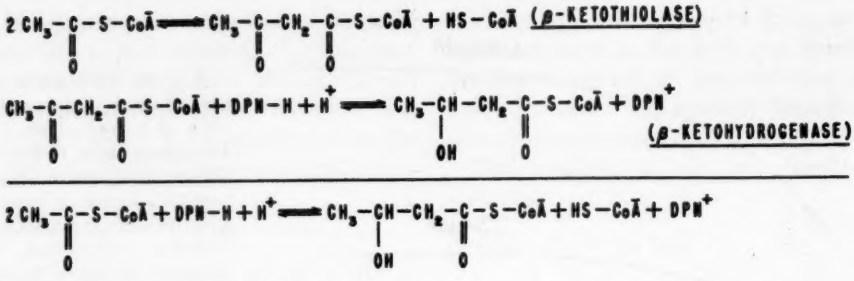


Fig. 15. β-KETOTHIOLASE TEST (26). Tris(hydroxymethyl)aminomethane-HCl buffer, pH 8.1, 200 μM; $MgCl_2$, 8.0 μM; reduced glutathione, 10 μM; CoA-SH, 0.15 μM; S-acetoacetyl CoA, ~0.03 μM. Volume, 1.5 ml; d = 0.5 cm; temperature, 25°.

for it requires a large amount of acetyl-CoA for each assay. But the more favorable back reaction, that is the thiolytic cleavage of acetoacetyl CoA, can be easily used. Here difficulties no longer exist since acetoacetyl CoA can now be readily prepared in several ways, including two purely chemical syntheses (15, 20, 21, 25). Figure 15 shows such a test, as used by Ochoa and Harting for the purification of this enzyme (26).

These are the four tests with the help of which the investigations of the various steps of the fatty acid cycle and the purification of the respective enzymes has become relatively simple (15, 19, 23, 26).

It is impossible to discuss all the experiments which have been done in my laboratory in Munich. Only one observation may be mentioned which concerns the enzyme of condensation,



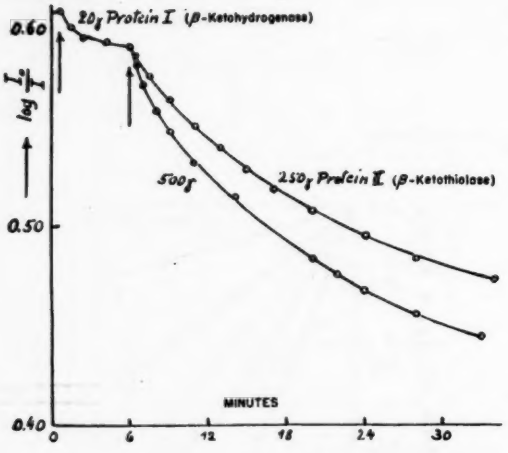
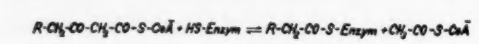


Fig. 16. β-KETOTHIOLASE TEST by coupling with β-keto reductase (15). Phosphate buffer, pH 6.4, 100 μM; ethylenediamine tetraacetate, 20 μM; acetyl-CoA, 0.35 μM; DPNH, 0.13 μM. Volume, 1.7 ml; λ, 366 mμ; d = 1 cm; temperature, 18°. At the first arrow 20 γ β-keto reductase (= β-ketohydrogenase), at the second 250 γ or 500 γ crude β-ketothiolase added.



'β-keto-thiolase,' as we named it, because of the position of the equilibrium. When we started work on this enzyme in Munich, acetoacetyl CoA was not yet available on a preparative scale. We therefore used, at that time, a coupled optical assay, in which we combined the action of keto thiolase with the action of keto reductase. In this test we start with acetyl-CoA and measure the decrease in DPNH absorption. An experiment of this kind is shown in figure 16, where it may be seen that the ΔE during the first few minutes is proportional to the amount of β-keto thiolase added. This procedure can therefore also be used for enzyme assay.

During these studies on β-ketothiolase we observed an interesting phenomenon. In testing

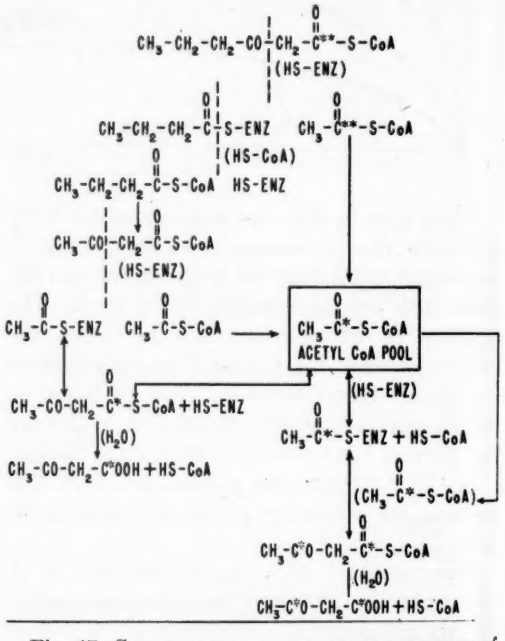


Fig. 17. SCHEME FOR UNEQUAL DISTRIBUTION of carbonyl carbon of caproic acid in acetoacetate.

several groups of inhibitors, we found that SH-poisons, like iodoacetate and arsenoxide, strongly inhibit the enzyme (14, 21). β-keto thiolase therefore belongs to the class of SH-enzymes (27). Now we believe that this SH-group, as integral part of the enzyme, participates in the chemical reaction, shown below:

This kind of enzyme action, with the active participation of the enzyme SH-group, would not only offer an explanation of the inhibition by iodoacetate and As_2O_3, but also of the unequal distribution of labeled acetyl-units in acetoacetic acid. In animal tissues the carboxyl carbon of fatty acids has a strong tendency to appear in the carboxyl group of acetoacetate, the penultimate carbon, however, appears, preferentially in the carbonyl group of this acid (28). An observation which forced Gurin *et al.* (29) to the assumption that two different active C_2 units arise during oxidation of fatty acids. In a reaction of this

kind, such would be the case. All C_2 units of the chain would form acetyl-CoA directly with the exception of the tail end of the fatty acid chain, which at first yields acetyl enzyme and then by further reaction with free CoA might also be transformed to acetyl-CoA. However, there is another possibility. It might also—and this is the reason for the unequal distribution—recondense directly with an acetyl-CoA unit of the pool and will then be preferentially located in the terminal position of acetoacetyl CoA. This mechanism is illustrated in figure 17 for the oxidation of carboxyl labeled caproic acid.

Through the kind invitation by Dr. Ochoa to his laboratory in New York, and through the generous grant of the Rockefeller Foundation, I got the opportunity to prove this hypothesis in a more direct way. I prepared S^{35} labeled CoA. By incubating this labeled CoA with unlabeled propionyl CoA in the presence of highly purified β-ketothiolase (26), I found a rapid uptake of label into the propionyl CoA. This, one would expect, is in agreement with the proposed mechanism; the transfer reaction

$$\text{propionyl} - \text{CoA} + \text{enzyme} \rightleftharpoons \text{propionyl enzyme} + \text{CoA}$$

occurs.

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DISCUSSION

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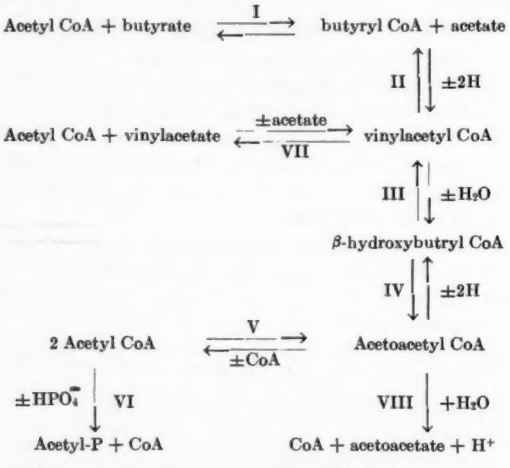
STUDIES with enzyme preparations derived from *Clostridium kluyveri* have shown that the mechanism of fatty acid oxidation in this organism is basically almost identical to that just described by Dr. Lynen for higher animals. The pathway of butyrate oxidation by extracts of *C. kluyveri* is represented by the following scheme:



This reaction scheme is essentially the same as that postulated by Barker (1) several years ago on the basis of circumstantial evidence, and has since received experimental support from studies in our laboratory (STADTMAN AND KORKES, unpublished results). According to this scheme, in the presence of orthophosphate (when *reaction VIII* is not involved), the oxidation of butyrate is a cyclic process in which CoA and acetyl CoA have catalytic roles, and in which butyryl CoA, vinylacetyl CoA, and acetoacetyl CoA are intermediates. In the course of one complete cycle, one mole of butyrate is oxidized, and one mole of acetate (*reaction I*) and one mole of acetyl P (*reaction VI*) are produced. Since only catalytic amounts of CoA and acetyl CoA are available, the continued regeneration of these substances is obligatory to the smooth operation of the cycle. This is accomplished through the combined actions of the two enzymes, β-ketothiolase and phosphotransacetylase. The β-ketothiolase (cf. LYNEN *et al.* (2)) catalyzes the thiolytic cleavage of acetoacetyl CoA by free CoA to form 2 molecules of acetyl CoA (*reaction V*). Under the influence of phosphotransacetylase, one of these acetyl CoA molecules is decomposed with orthophosphate to form acetyl-P (*reaction V*), thus regenerating CoA which is available for re-use in *reaction V*. The other molecule of acetyl CoA derived from acetoacetyl CoA cleavage is used for the formation of butyryl CoA (*reaction I*) needed to start the cycle over again.

The coenzyme A transphorase catalyzing *reactions I* and *VII* (3) and the phosphotransacetylase catalyzing *reaction V* (4) have been partially purified and studied in some detail. Further evidence for the scheme has been obtained from studies with an enzyme preparation that will not catalyze appreciable oxidation of butyrate in the absence of orthophosphate unless substrate amounts of CoA are added. This preparation is therefore presumed to be deficient in the hydrolyase catalyzing the hydrolysis of acetoacetyl CoA (*reaction VIII*). With such a preparation (in the absence of orthophosphate) synthetic butyryl CoA is oxidized with the uptake of one equivalent of molecular oxygen and acetoacetyl CoA has been isolated from the reaction mixture as a crude barium salt (cf. STERN AND COON (5)) and has been identified by the spectrophotometric method of Lynen *et al.* (2) as modified by Stern and Coon (5). It was further characterized as an acetoacetyl ester by treating with hydrazine under mild conditions to form the 3 methyl 5 pyrazolone derivative. Incubation of the isolated acetoacetyl CoA with an extract of *C. kluyveri* and free CoA results in the formation of acetyl CoA as indicated by *reaction V*.

The over-all *reaction II* through *V* is observed when butyryl CoA is oxidized in the presence of a stoichiometric amount of CoA. Under these conditions one mole of oxygen is consumed and 2 moles of acetyl CoA are formed per mole of butyryl CoA added. As is indicated by *reaction I*, a mixture of acetyl CoA and butyrate may be substituted for butyryl CoA.

No direct evidence for vinylacetyl CoA and β-hydroxybutyryl CoA as intermediates has been obtained in the bacterial studies. However, in view of the very beautiful work presented by Dr. Lynen at this symposium it appears probable that β-hydroxybutyryl CoA is an intermediate.

The suggestion that vinylacetyl CoA is an inter-

mediate in the oxidation of butyryl CoA by *C. kluyveri* is based upon the fact that vinylacetate is readily oxidized by the bacterial extracts whereas crotonate is not attacked. In view of the experiments just described by Lynen, indicating that crotonyl CoA is formed from β-hydroxybutyryl CoA in the animal enzyme system, the role of vinylacetyl CoA as the immediate oxidation product of butyryl CoA in *C. kluyveri* is subject to doubt. On the other hand, the experiments described by Mahler in this symposium showing that the animal hydrase catalyzes the formation β-hydroxybutyrate from both vinylacetyl CoA and crotonyl CoA indicate that under the influence of this enzyme these substances are interconvertible and may be in equilibrium with each other. The possibility that vinylacetyl CoA may be an intermediate in butyryl CoA oxidation is therefore still open.

Since the first step in the oxidation of a free fatty acid is an activation step, i.e., the formation of the acyl CoA derivative, it is possible that the inertness of crotonate in the bacterial system is due to the inability to synthesize crotonyl CoA by a reaction similar to *reactions I* and *VII*. This idea is supported by the finding that crotonate will not serve as a CoA acceptor in the CoA transphorase system.

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ROLE OF COENZYME A IN FATTY ACID METABOLISM[1]

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OUR UNDERSTANDING of the detailed biochemical events in the metabolism of the three major classes of nutrients—carbohydrates, proteins and fats—bears a direct relation to our ability to isolate, purify and study the enzymes involved. Not only is it necessary to investigate the individual steps but also to reconstruct the complete process which may show features not obvious in the individual steps themselves.

Highly purified preparations have been available for many years to the investigator interested in carbohydrate metabolism. The researches of several decades conducted by numerous groups of biochemists have culminated in the Embden-Parnas-Meyerhof scheme of glycolysis, and in an understanding of the interrelations between glucose and glycogen. More recently the interconversions of various sugar phosphates have been studied with purified enzyme systems.

Mitochondrial Fatty Acid Oxidation. On the other hand the gleanings for those of us interested in the metabolism of fatty acids have been meager indeed, except for the last decade. For it was only in 1943 that the first cell-free preparation capable of oxidizing fatty acids was described by Leloir and Muñoz (1).[2] The period from 1943 to 1951 saw several significant strides taken toward the goal of a complete analysis of the fatty acid metabolizing system. The mitochondrion was identified as the locale of oxidation (2), particulate preparations and their mode of action were studied in great detail (3), and the eventual fate of tracer carbon originally incorporated in the fatty acid molecule was established (4).

It may be well to pause at this point and to summarize the essential facts which had emerged from studies carried out on particulate systems. Any theory of fatty acid oxidation based on experiments with isolated enzymes must be consistent with and provide an explanation for the conclusions based on work with the particulate systems:

a) Fatty acids are not metabolized as such. They must first be converted to a metabolically active form of unknown structure. The process of oxidative phosphorylation which accompanies co-oxidation of a catalytic amount of a citric-acid cycle substrate appears to provide the mechanism for the initiation of fatty acid oxidation. When oxidative phosphorylation is inhibited, fatty acid oxidation also ceases. This initiation of fatty acid oxidation by concomitant oxidation of another substrate has been called the sparking or priming phenomenon.

b) Under ordinary circumstances the products of fatty acid oxidation are carbon dioxide and water in most tissues. In liver, especially in the presence of malonate, acetoacetate is also an end product of oxidation. It is not, however, a true intermediate in the oxidative conversion of substrate to carbon dioxide, since it is metabolically inert.

c) The process of β-oxidation, first discovered by Knoop, provides the pathway for fatty acid breakdown. The two-carbon fragments so formed are on the oxidation level of acetate, but are not identical with acetate. Heart or kidney mitochondria can convert acetate to its active form providing oxidation of any member of the citric acid cycle is taking place concurrently.

d) The 'active acetate' formed by β-oxidation is further metabolized by condensation with oxalacetate to yield citrate, which in turn is completely oxidized by way of the Krebs-citric acid cycle. Alternatively, a pair of two-carbon units may condense to yield acetoacetate.

e) The various postulated intermediates be-

[1] The research reported here has been carried out by Drs. H. Beinert, R. M. Bock, Dexter S. Goldman, D. E. Green, Sanae Mii, P. G. Stansly, S. J. Wakil and the author. It was supported by grants from the National Heart Institute, National Institutes of Health, and the American Cancer Society (on recommendation of the Committee on Growth, National Research Council), and by a contract with the U. S. Atomic Energy Commission, Division of Biology and Medicine.

[2] In the interest of brevity, references to work on fatty acid oxidation prior to 1951 will be to only a few reviews or long papers. No effort has been made to include all the publications in this very active field.

tween the starting fatty acid and active acetate do not accumulate, nor can they be identified by conventional isotope techniques. On the other hand these compounds, i.e. the α,β-unsaturated acids, the β-hydroxy acids and the β-keto acids are rapidly metabolized by the enzyme system, at rates comparable to those of the saturated fatty acids.

f) If carboxyl labeled acids are used and the acetoacetate formed is isolated and degraded the ratio of label in the carbonyl carbon: label in the carboxyl carbon varies in a regular manner with chain length rising from about 0.3 for butyrate, to 0.8 for octanoate, and to 1.0 for higher fatty acids. Terminally labeled acids give rise to acetoacetate labeled predominantly in the carbonyl fragment.

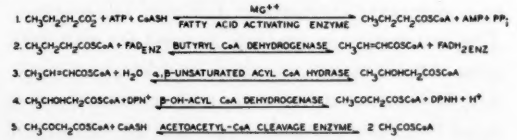
Role of Acetyl CoA. Three important lines of discovery finally brought the field to the point where complete reconstruction of the enzymatic steps appeared feasible. Stadtman and Barker discovered that soluble extracts of *Cl. kluyveri* catalyzed the aerobic oxidation of butyrate or caproate to acetyl phosphate and the anaerobic syntheses of caproate or butyrate from alcohol and acetate (5). Their elegant work, and the subsequent investigations of Stadtman on phosphotransacetylase led Barker and his colleagues to a speculative formulation of the steps in fatty acid oxidation (6) which later work was to bear out in its essentials. Concurrently, Lipmann, Novelli and their group were studying the phenomenon of acetylation and acyl transfer, and established the function and structure of coenzyme A. They showed that the coenzyme acted as the vehicle not only for the transfer of acyl groups but also for the generation of these groups from short-chain fatty acids in the presence of ATP[3] or acetyl phosphate (7). Finally, Lynen's brilliant tour de force of isolating the elusive 'active acetate' of the biochemical literature and its identification as S-acetyl CoA (8) opened the door to a host of new theoretical and practical advances.

[3] The following abbreviations will be used throughout this presentation: ATP, adenosinetriphosphate; AMP, adenosine-5'-phosphate; PP_i, inorganic pyrophosphate; CoA or CoASH, reduced coenzyme A; DPN or DPN^+, diphosphopyridine nucleotide; DPNH, reduced DPN; TPN, triphosphopyridine nucleotide; FAD, flavin adenine dinucleotide; $FADH_2$, reduced FAD; FAD_{enz}, $FADH_{2enz}$, enzyme-bound flavins; TTZ, triphenyltetrazolium; Ac, acetyl; AcAc, acetoacetyl.

Reconstruction of Fatty Acid Oxidizing System of Animal Tissues. Our own laboratory had been concerned with fatty acid oxidation for several years (9). After covering several aspects of this problem in the cyclophorase-mitochondrial system, we next turned to a heart muscle preparation which consisted of a soluble and a particulate enzyme fraction. All the fatty acid oxidizing enzymes proper were present in soluble form, while the succinoxidase system and the electron transport system to oxygen were still attached to particles (10). By the use of this preparation, we could clearly establish the requirement for coenzyme A, ATP and DPN, but still could not subdivide the process into its component enzymatic steps or establish the nature of the intermediates.

The next move, clearly, was to eliminate the necessity for the electron transport system to oxygen and to study the process of fatty acid oxidation by means of soluble enzymes exclusively. Using DPN and triphenyltetrazolium as electron acceptors, we were able to study butyrate oxidation in the soluble fraction of the heart muscle preparation (11). From these studies the requirement for more than one enzymatic component in the conversion of butyrate to acetoacetate, as well as for the cofactors CoA, DPN, ATP and Mg^{++} clearly emerged.

In the meantime three developments had occurred which facilitated more rapid progress: Drysdale (12) reported on a soluble active fatty acid oxidase system obtained from rat liver mitochondria. In our laboratory methods were developed for the large scale isolation of coenzyme A from yeast and for the preparation of active mitochondrial suspensions in kilogram lots from slaughterhouse tissue. With such preparations as starting materials, we have been able to identify, isolate and bring to a high state of purity all the enzymes concerned in the conversion of fatty acids to acetyl CoA. The reactions catalyzed and the enzymes concerned in the case of butyrate are:


1. $CH_3CH_2CH_2CO_2^- + ATP + CoASH \xrightarrow[\text{FATTY ACID ACTIVATING ENZYME}]{Mg^{++}} CH_3CH_2CH_2COSCoA + AMP + PP_i$
2. $CH_3CH_2CH_2COSCoA + FAD_{ENZ} \xrightarrow{\text{BUTYRYL CoA DEHYDROGENASE}} CH_3CH{=}CHCOSCoA + FADH_{2ENZ}$
3. $CH_3CH{=}CHCOSCoA + H_2O \xrightarrow{\alpha,\beta\text{-UNSATURATED ACYL CoA HYDRASE}} CH_3CHOHCH_2COSCoA$
4. $CH_3CHOHCH_2COSCoA + DPN^+ \xrightarrow{\beta\text{-OH-ACYL CoA DEHYDROGENASE}} CH_3COCH_2COSCoA + DPNH + H^+$
5. $CH_3COCH_2COSCoA + CoASH \xrightarrow{\text{ACETOACETYL-CoA CLEAVAGE ENZYME}} 2\ CH_3COSCoA$

It can be seen that activation of fatty acids can be equated with their conversion to the S-acyl CoA derivatives, which are the true substrates of the enzymes concerned in the various

subsequent conversions. The whole sequence, albeit at the acyl CoA level, bears some resemblance to that responsible for the transformations from succinate to oxalacetate in the citric acid cycle.

Activation (13).[4] The enzyme, catalyzing *reaction 1*, i.e. the activation step, has been purified by the following sequence of operations: beef liver mitochondria → acetone powder → phosphate extract → first $(NH_4)_2SO_4$ fractionation → second fractionation with $(NH_4)_2SO_4$ → treatment with alumina C_γ → third fractionation with $(NH_4)_2SO_4$ to a stage where it is homogeneous in the analytical ultracentrifuge with a sedimentation constant (S_{20w}) $\simeq$ 4. Preparations

TABLE 1. SPECIFICITY OF FAAE[1]

	Rate[2] (ΔD_{320}/10 min. x 10^3)		Rate (ΔD_{320}/10 min. x 10^3)
Saturated Fatty Acids		*Unsaturated Fatty Acids*	
C_2	000	$\Delta^{2,3}$ C_4	100
C_3	000	$\Delta^{3,4}$ C_4	90
C_4	150	$\Delta^{2,3}$ C_6	270
C_5	250	$\Delta^{2,3,4,5}$ C_6	300
C_6	280		
C_7	325	*β-Hydroxy Fatty Acids*	
C_8	225	C_4	50
C_9	185	C_6	140
C_{10}	150	C_8	125
C_{11}	50	C_{12}	110
C_{12}	000		

[1] The system contained 25 μM 2-amino-2-methyl-1,3-propane diol buffer (pH 9.0), 1.1 μM ATP, 4.4 μM $MgCl_2$, 0.17 μM CoASH, 14 μg enzyme and 2 μM of the substrates shown, in 0.20 ml. The blank contained all components except the substrate. Incubation time, 10 min. at 38°.

[2] 1 μ mole CoASH = O.D. of 2.1 at 520 mμ.

of this stage of purity show more than one peak on electrophoresis. The main peak, comprising approximately 60% of the total protein, however, carries all the enzymatic activity.

Several different methods may be used for measuring the activity of the enzyme. The one ordinarily employed in enzyme purification depends upon the disappearance of the free sulfhydryl group of coenzyme A during the reaction. The nitroprusside reaction is used for this purpose. An alternative method found to be equivalent to the first mentioned, and superior to it in some respects, depends upon the appearance of S-acyl groups which can be measured spectrophotometrically at 232 mμ. Using these methods the stoichiometry of the reaction has been found to be in conformity with *equation 1*. In a typical experiment the disappearance of 1.00 μ mole of CoASH and of 0.92 μ mole ATP was accompanied by the appearance of 1.08 μ mole butyryl CoA, 0.96 μ mole of PP_i and 1.05 μ mole of AMP.

A wide variety of fatty acids tested was acted upon by the enzyme, as can be seen from table 1. In addition to the acids mentioned in the table, branched chain, and phenyl substituted acids are also acted on by the enzyme. Dicarboxylic or amino acids are completely inactive.

Reaction 1 was found to be strictly reversible, with an equilibrium constant essentially equal to unity. From this measurement and the known ΔF for the reaction ATP = AMP + PP_i it is, therefore, apparent that the acyl CoA derivatives are 'high-energy' compounds with a free energy of hydrolysis for *reaction 6* of approximately 11,000 calories:

6. $RCOSCoA + H_2O = RCO_2H + CoASH$
$\Delta F^\circ \simeq -11{,}000$ cal

The wide range of activity of the enzyme has made it an invaluable tool in the study of the subsequent reactions undergone by the acyl CoA derivatives. It has provided a means for the synthesis of all the various acyl CoA's required.

Dehydrogenation by Flavin (14, 15). The first oxidative step in butyrate metabolism is the conversion of butyryl to butenoyl CoA. It is catalyzed by an enzyme, brillliantly green in color, which has been purified by the following series of steps: extract of beef liver mitochondria acetone powder → $(NH_4)_2SO_4$ fractionation → treatment with $Zn(OH)_2$ gel → first alcohol fractionation → adsorption on alumina C_γ → second alcohol fractionation → preparative electrophoresis at pH 8.0. Prior to the last step, the enzyme is approximately 50% pure, while the final step which removes all remaining contaminants is made feasible by the high electrophoretic mobility of the enzyme protein compared to the other impurities at that pH. The enzyme so isolated is homogeneous in the ultracentrifuge under a variety of conditions. It shows an absorption spectrum similar to that of flavoproteins with the peaks, however, shifted towards shorter wavelengths, and, in addition, a fourth peak *not* shown by other flavoproteins in the range 680–685 mμ. The relative extinctions at the 4 maxima (wavelengths in mμ) $E_{265} : E_{355} : E_{431.5} : E_{680}$ are, 7.76 : 0.800 : 1.00 : 0.240.

The riboflavin content of the homogeneous enzyme is 1.20 weight per cent, which would

[4] For detailed references to the methods used in this and subsequent sections, the reader is referred to the appropriate publications dealing with the individual enzymes.

correspond to a minimal molecular weight (1 M flavin/M enzyme) of 31,000. The actual molecular weight as determined from sedimentation constant and boundary sharpening lies in the range 100,000 to 200,000—a fact which suggests that the actual enzyme molecule as isolated contains more than one flavin prosthetic group. By treatment with acid ammonium sulfate the enzyme can be converted to an apoenzyme, almost inactive in the 2,6-dichlorophenolindophenol test system employed. Five-fold stimulation of activity, corresponding to complete restoration of initial activity, can be obtained by the addition of FAD or a boiled extract of the purified enzyme. The latter shows an absorption spectrum identical with that of FAD and is equally effective as the dinucleotide in the D-amino acid apooxidase assay.

Several methods have been used for the direct measurement of the enzymatic activity of the dehydrogenase. Since the pure flavoprotein can be reduced by dithionite, borohydride or by butyryl CoA, changes in extinction in the range 425–440 mμ provide a means of studying the direct interaction of substrate and flavoprotein in a Beckman recording spectrophotometer. The enzyme is reduced in less than 3 seconds by butyryl CoA but not by butyrate. Octanoyl CoA is incapable of effecting this rapid reduction. The reduced flavoprotein can be rapidly reoxidized by the addition of crotonyl CoA.

Usually enzymatic activity has been measured by means of an oxido-reduction dye of appropriate potential, such as 2,6-dichlorophenolindophenol, according to *equations 2*, *7* and *8*.

$$8 = \frac{7 \quad FADH_{2enz} + \text{indophenol} = FAD_{enz}}{2 + 7 \quad CH_3CH_2CH_2COSCoA + \text{indophenol} = CH_3CH{=}CHCOSCoA + \text{indophenol } H_2}$$

The rate of dye reduction is zero-order and provides a linear measure of enzyme concentration. Using this assay system, the range of specificity of the enzyme has been established as extending from propionyl CoA to heptanoyl CoA with the following relative activities: $C_3 = 25$, $C_4 = 100$, $C_5 = 55$, $C_6 = 45$, $C_7 = 35$. The indophenol system has also been used for the determination of the exact nature of the product of *reaction 8*. As will be shown, in the absence of hydrase (the enzyme catalyzing *reaction 3*), only β-hydroxyacyl CoA's are acted on by the DPN-requiring dehydrogenase effecting *reaction 4*. The product of *reaction 8* is completely inert in the presence of DPN, diaphorase (16), and dehydrogenase. If purified hydrase is also added, DPN is reduced rapidly and in presence of diaphorase indophenol reduction can also be observed. The reaction product is, therefore, crotonyl CoA.

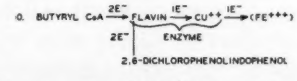
After solutions of the green flavoprotein have been dialyzed exhaustively and then subjected to analytical arc and spark spectroscopy, the presence of copper tightly bound to the enzyme can be demonstrated. The copper content of the protein (measured accurately by a manometric method after exhaustive dialysis and ashing of the preparation) increases during purification but the molecular ratio copper:flavin remains constant and has a value of 2.

The absorption spectrum of the enzyme undergoes profound changes on treatment with butyryl CoA. The 3 peaks in the visible are reduced to approximately 0.5 their original value. If the enzyme in buffer of pH 7.5 is dialyzed vs. 5×10^{-3} M KCN for 36 hours, the peak at 685 mμ disappears and the enzyme now shows a typical three banded flavoprotein spectrum.

The cyanide-treated enzyme is still reducible by butyryl CoA (as determined by changes in the region 390–480 mμ) and its catalysis of *reaction 8* is unimpaired. Thus, interaction with oxidizing agents involving two-electron changes is unaffected by removal of copper. On the other hand, when the enzyme reacts with oxidizing agents which go through a one electron change such as ferricytochrome *c* or ferricyanide,

$$9.\ \text{butyryl CoA} + 2(Fe^{+++}) \rightarrow \text{crotonyl CoA} + 2(Fe^{++}) + 2\ H^+$$

the rates of reduction as catalyzed by the cyanide treated enzyme are markedly lowered (to 0.5–0.3 times the original). Full activity can be restored by preincubation of the enzyme with 5×10^{-3} M $CuSO_4$. Thus it may be permissible to postulate the following scheme for the various inter- and intramolecular oxidoreductions mediated by the enzyme.



Hydration, and Dehydrogenation by DPN (14, 17). It may be well to discuss the enzymes catalyzing *reactions 3* and *4* concurrently, not only because their methods of purification are quite similar, but also since the assay for the hydrase (*reaction 3*) involves the use of the dehydrogenase (*reaction 4*).

The β-hydroxyacyl CoA dehydrogenase has been isolated from beef liver mitochondria

through steps quite analogous to those already described for the previous enzymes and involving extraction of the acetone powder → first $(NH_4)_2SO_4$ fractionation → adsorption on and elution from alumina C_γ → second $(NH_4)_2SO_4$ fractionation → alcohol fractionation → third $(NH_4)_2SO_4$ fractionation. The assay system is based on the reduction of DPN spectrophotometrically, with β-hydroxyacyl CoA as the substrate at pH 9. Under those conditions initial reduction rates are linear and proportional to enzyme concentration. All the hydroxyacyl CoA's from C_4 to C_{12} which have been tested are oxidized at approximately the same rate. At the highest purity level so far obtained 1 mg of the enzyme catalyzes the oxidation of 200 μ moles substrate/min. at 22° and pH 9. DPN can be replaced by coenzyme III (18), but not by TPN. Using crude enzyme preparations which catalyzed the oxidation of β-hydroxy-butyrate by DPN in the presence of ATP, Lehninger and Greville found that the *d*-isomer was the one oxidized in the presence of the enzyme (19). This has been borne out by studies with highly purified enzymes. The *d,l* and *l*-hydroxybutyryl CoA derivatives were prepared by means of the activating enzyme (*equation 1*) and isolated. When tested with the dehydrogenase the *l*-enantiomorph was found to be completely inert, while the *d,l* mixture showed the customary activity.

We have measured the equilibrium constant for *reaction 4* and found that $K_4 = ([\beta\text{-keto}][DPNH][H^+])/([\beta\text{-OH}][DPN])$ has a value of 6.3×10^{-11} moles/liter. From this and the value for the DPN-DPNH couple, the E_0' for the couple β-hydroxybutyryl CoA/acetoacetyl CoA can be calculated and is found to be −0.224 V. It can be seen that this value is approximately 50 mV higher than that generally accepted for the couple β-hydroxybutyrate-acetoacetate. In this connection it may be of interest to report that the couple butyryl CoA-crotonyl CoA has an E_0' equal to about +0.20 V. This value is considerably higher than the (calculated) value for butyrate-crotonate (20).

The hydrase, the enzyme catalyzing *reaction 3*, is a very persistent contaminant of most preparations of the dehydrogenase. However, by careful fractionation techniques, preparations of the two enzymes free from cross-contamination can be obtained. By the use of these preparations and the DPN-assay already described for the dehydrogenase, it has been established that the reaction catalyzed by the hydrase is the one summarized in *equation 11*.

11. $RCH{=}CHCH_2COSCoA \xleftarrow{H_2O} d{-}RCH_2CHOHCH_2COSCoA \xrightarrow{H_2O} RCH_2CH{=}CHCOSCoA$

Thus, at least in the case of the C_4 acids, both vinyl acetyl CoA and crotonyl CoA are converted to *d*-hydroxybutyryl CoA. All the α,β unsaturated acyl CoA's tested from C_4 to C_{12} were active. As already stated, the enzymes catalyzing the reactions just described are only active with the acyl CoA derivatives. The free acids themselves are completely untouched by the appropriate enzymes. The equilibrium ratio, unsaturated acyl CoA/β-OH acyl CoA, for C_4 acids is about 0.5.

The product of *reaction 4* has been isolated from the incubation mixture by means of phenol-benzyl alcohol extraction and has been identified as acetoacetyl CoA on the basis of the following evidence: *a*) in the presence of DPNH and β-hydroxyacyl CoA dehydrogenase, reoxidation of the nucleotide can be observed at pH 6.0 (17); *b*) in the presence of cleavage enzyme and free SH CoA, one equivalent of SH is consumed and two of acetyl CoA are produced (20) according to the stoichiometry of *equation 5*; *c*) free keto acid can be liberated (and estimated by conventional means) after mild alkaline hydrolysis or by incubation with succinate and succinyl CoA-acetoacetate transferase (21, 22) according to *equation 12*.

12. Acetoacetyl CoA + succinate ⇌ succinyl CoA + acetoacetate

In a typical experiment 0.12 μ moles of acetoacetyl CoA (determined as acetoacetate after hydrolysis) was converted to 0.23 μ moles of citrate, in the presence of CoASH, malate, malic dehydrogenase (23) and condensing enzyme (24) with the simultaneous reduction of 0.24 μ moles of DPN. This stoichiometry is the one required by *reaction 15*.

5. Acetoacetyl SCoA + CoASH ⟷ 2 acetyl SCoA

13. $2\ Malate^= + 2\ DPN^+ \xleftarrow{\text{malic dehydrogenase}} 2\ oxalacetate^= + 2\ DPNH + 2\ H^+$

14. 2 Oxalacetate$^{=}$ + 2 acetyl CoA +

$$2\ OH^- \xleftarrow{\text{condensing enzyme}} 2\ \text{citrate}^{\equiv} + 2\ \text{CoASH}$$

15. Acetoacetyl SCoA + 2 malate$^{=}$ +

$$2\ DPN^+ + 2\ OH^- \longleftrightarrow 2\ \text{citrate}^{\equiv} + 2\ DPNH + 2\ H^+ + \text{CoASH}$$

Similar experiments have also been performed with the higher homologues of acetoacetyl CoA.

Cleavage to Acetyl CoA (17, 20, 21, 22). For the assay of the cleavage enzyme (*reaction 5*), the enzymes catalyzing *reactions 3, 4, 13* and *14* are used in excess in the presence of crotonyl CoA (synthetically prepared), DPN, a catalytic amount of free CoA, and malate. Then the reduction of DPN according to *equation 16* is measured.

16. (sum of *3, 4, 5, 13* and *14*)

$$\text{Crotonyl CoA} + H_2O + 3\ DPN^+ + \text{malate}^{=} + 2\ OH^- \longleftrightarrow 2\ \text{citrate}^{\equiv} + \text{CoASH} + 3\ DPNH + 3\ H^+$$

A highly purified preparation of the cleavage enzyme has been obtained from beef liver mitochondria through the steps: acetone powder extract → neutral $(NH_4)_2SO_4$ fractionation → first alcohol fractionation → alkaline $(NH_4)_2SO_4$ fractionation → second alcohol fractionation. An enzyme isolated in this manner was found to function with all the β-ketoacyl CoA derivatives between C_4 and C_8 which have been tested.

The reactions of *equations 4* and *5* can also be followed by observing changes in acyl CoA concentration as measured by the hydroxamic acid reaction. β-Ketoacyl CoA's do not form the hydroxamates so characteristic of other acyl CoA derivatives. In one experiment, when 0.25 μ moles of β-hydroxybutyryl CoA were oxidized according to *equation 4*, 0.25 μ moles of DPNH were formed and 0.25 μ moles of material reacting with NH_2OH disappeared. There was no appearance of free sulfhydryl. On addition of cleavage enzyme and free CoASH, 0.47 μ moles of a compound reacting with NH_2OH was formed and 0.24 μ mole of CoASH disappeared, in good agreement with the stoichiometry required by *reactions 4* and *5*.

Thus it has been possible to convert butyrate to citrate in a step-wise manner and the detailed enzymatic mechanism of butyrate oxidation has been found to conform to the postulations of *equations 1* through *5*. It has also been feasible to study the overall conversion by means of an assay system in which the individual oxidation steps are linked to the oxido-reduction dye triphenyltetrazolium and which is based on the appearance of the reduced formazan. The additional steps necessary are those summarized in *equations 17, 18* and *19*.

TABLE 2. CONVERSION OF BUTYRATE TO CITRATE (14)

	Formazan μM/hr		Formazan μM/hr
Complete system	0.308	Complete system	0.336
no fatty acid activating enzyme	0.003	no malate	0.000
no butyryl CoA dehydrogenase	0.004	no ATP	0.017
no hydrase, β-OH acyl CoA dehydrogenase, or cleavage enzyme	0.000	no DPN	0.000
no diaphorase	0.012	no CoA	0.000
no condensing enzyme	0.008	no methylene blue	0.000

The complete system contained fatty acid activating enzyme (20γ); a mixture of hydrase, β-OH acyl CoA dehydrogenase and cleavage enzyme (20γ); diaphorase (10γ); malic dehydrogenase (10γ); condensing enzyme (4γ); malate (0.75 μM); ATP (0.4 μM); DPN (0.1 μM); CoA (0.08 μM); methylene blue (0.1 μM); and TTz (4 μM). Final volume 0.3 ml; incubated at 38°.

17. $FADH_{2enz}$ +

$$\text{methylene blue} \xleftarrow{\text{butyryl CoA dehydrogenase}} FAD_{enz} + \text{methylene blue } H_2$$

18. Methylene blue H_2 + TTZ ⟷ methylene blue + $TTZH_2$

19. DPNH + methylene blue +

$$H^+ \xleftarrow{\text{diaphorase}} DPN^+ + \text{methylene blue } H_2$$

Using this assay system results such as those shown in table 2 were observed.

Oxidation of Octanoate. When we next attempted the complete oxidation of higher fatty acids such as octanoate, we found that a system containing all the enzymes so far described was incapable of carrying out this oxidation in the absence of an additional enzymatic component. This enzyme, yellow in color, was found to

substitute for the green butyryl CoA dehydrogenase in the case of the higher fatty acids, i.e. it is a more general acyl CoA dehydrogenase.

TABLE 3. SPECIFICITY OF GREEN AND YELLOW ACYL CoA DEHYDROGENASES

Relative Activity (ΔD_{600}/5 min. $\times$ 10^3)

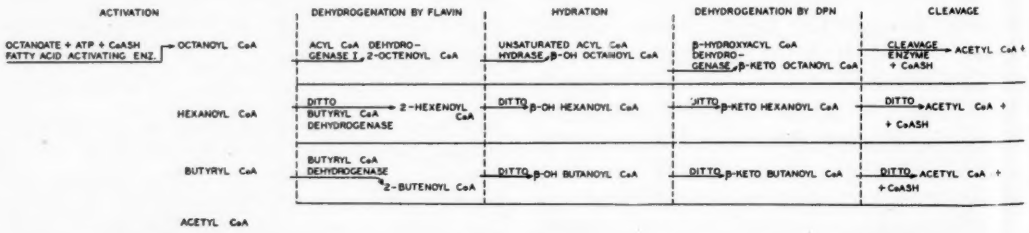
Fatty Acid	butyryl CoA dehydrogenase	acyl CoA dehydrogenase	acyl CoA dehydrogenase (corrected)
C_3	20	0	0
C_4	100	100	0
C_5	55	70	15
C_6	40	70	30
C_7	30	95	65
C_8	8	100	92
C_{10}	0	100	100

All cuvettes contained enzyme (15γ butyryl CoA dehydrogenase of 100γ acyl CoA dehydrogenase, not free of butyryl CoA dehydrogenase), Tris buffer pH 7.0, 20γ 2,6 dichlorophenol indophenol, and 100 millimicromoles of acyl CoA.

Table 3 shows the relative activities of the two dehydrogenases when tested with different acids from C_3 to C_{10}. By the use of this enzyme plus the ones already described we could observe the step-wise transformations summarized in Scheme 1.

SCHEME I

ENZYMATIC OXIDATION OF OCTANOATE



Accumulation of Intermediates. We are inclined to believe that the scheme just proposed, taken in conjunction with the properties of the individual enzymes described, accounts for all the observations on particulate systems discussed earlier. A few additional comments concerning the accumulation of intermediates and some of the isotope experiments may, however, be in order.

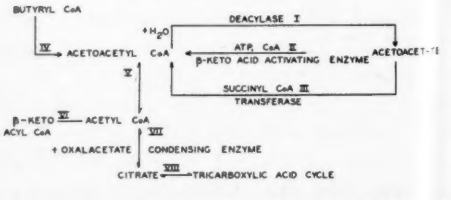
The true intermediates in fatty acid oxidation are the acyl CoA derivatives. All the acids in the scheme, from the starting substrate to C_2, are always bound to the coenzyme in thioester linkage; the whole sequence of β-oxidation and cleavage of 2 carbon units is operative for acyl CoA derivatives only. Thus the total concentration of true intermediates of fatty acid oxidation can never exceed and is probably considerably lower than the total concentration of (firmly bound) coenzyme A in the mitochondrion, i.e. about 1 mg/g. dry liver mitochondria.[5] It will be remembered that mitochondrial oxidation proceeds optimally in the complete absence of any added CoA.

What are the relative steady state concentrations of the various acyl CoA's? We can arrive at some tentative conclusions from a consideration of turnover numbers of the several enzymes involved. The approximate turnover numbers of the enzymes (moles substrate metabolized $\times$ mole^{-1} enzyme $\times$ min^{-1} at 38°) in butyrate oxidation, assuming molecular weights of 100,000 for all enzymes and equimolar concentrations, are: 200 for activation, 500 for the first oxidation, 20,000 for hydration, 10,000 for the second oxidative step, and 700 for cleavage. It follows that the β-ketoacyl CoA derivatives are the one group of compounds in the whole sequence which are formed at a much greater rate than they are metabolized. The largest proportion of the CoA molecules concerned in fatty acid oxidation should, therefore, be tied up as the β-ketoacyl derivatives at any one moment and in the absence of any competing reactions.

Why then does only acetoacetate itself accumulate in liver and why is added acetoacetate metabolically inert in this tissue? Scheme 2 shows

SCHEME II

ACCUMULATION OF ACETOACETATE



the known enzymatic conversions which operate in the metabolism of acetoacetate: Acetoacetyl

[5] Experiments of L. J. Teply quoted in GREEN, D. E. *Biol. Rev.* 26: 410, 1951.

CoA is continuously being formed in the reactions numbered *4*, and *6* plus *5*; it is used up reversibly in *reactions 5* and *7*; but it is irreversibly transformed to acetoacetate in *reaction I*. In liver extracts there is present an exceedingly active deacylase (*reaction 1*) specific for acetoacetyl CoA, while the β-keto acid activating enzyme (*reaction 2*) which can form acetoacetyl CoA again from acetoacetate at the expense of ATP is very weak.[6] Furthermore, the transferase (*reaction 3*) which accomplishes the activation of acetoacetyl CoA at the expense of succinyl CoA is completely absent[6] (20). Thus the amount of acetoacetate accumulating will be governed by the relative rates of acetoacetyl CoA disappearance through *5* and *1*. Anything interfering with *5* will increase the proportion irreversibly degraded to free acetoacetate. Malonate accomplishes just that.

TABLE 4. INCORPORATION OF LABEL FROM Ac*CoA INTO $AcAcO^-$ IN PRESENCE OF VARYING AMOUNTS OF ADDED $AcAcO^-$ (25)

Experiment No.	Incubation		Ac*CoA Added[1]	$AcAcO^-$ added	C^{14} recovered in $AcAcO^-$ cpm		$\frac{C^*O}{C^*OOH}$
	Time	Temp.			CO	COOH	
	min.	°C.	μM	μM			
1	10	30	0.5	0	460	460	1.00
	10	30	0.5	0.4	13,050	21,100	0.62
	10	30	0.5	3.2	12,200	26,700	0.46
2	10	30	0.5	0.46	9,460	13,550	0.70
	10	30	0.5	3.2	9,130	15,800	0.58
	10	30	0.5	25.0	11,370	22,600	0.50
	10	30	0.5	125.0	7,000[3]	15,990[3]	0.44
	100	30	0.5	0.46	15,290	17,155	0.89
	100	30	0.5	3.2	16,050	27,300	0.59
3	12	38	2.0	3.0	11,300	19,650	0.57
	12	38	2.0	30.0	10,600	28,200	0.38
4[2]	8	38	2.0	3.0	365	400	0.91

[1] Total radioactivity in each sample was adjusted to 75,000 cpm. [2] No enzyme was added in this experiment. [3] Low values are probably due to inhibition of ACE in presence of high concentration of $AcAcO^-$.

Isotope Distribution (25). As far as isotope distribution is concerned, it has been possible to reproduce the effects observed in particulate preparation by means of a soluble system containing only labeled acetyl CoA, acetoacetate, transferase and cleavage enzyme.[7] Table 4 shows some of the results observed. The findings have been explained in the following manner:

20. $E_{cl} + Ac^*CoA \rightleftharpoons E_{cl} - Ac^*CoA$

21. $E_{cl} - Ac^*CoA + Ac^*CoA \rightleftharpoons E_{cl} - Ac^*Ac^*CoA + CoA$

Ac^*Ac^*CoA then dissociates from the cleavage site, combines with the transferring or replacement site of the ACS, and in the presence of unlabeled $AcAcO^-$ a *reaction 22* analogous to *reaction 12* leading to unlabeled acetoacetyl CoA takes place:

22. $Ac^*Ac^*CoA - E_{Re} + AcAcO^- \rightleftharpoons AcAcCoA - E_{Re} + Ac^*Ac^*O^-$

After another change of sites, AcAcCoA may then undergo *reaction 23*, the reverse of *reaction 21*:

23. $E_{cl} - AcAcCoA + CoA \rightleftharpoons E_{cl} - AcCoA + AcCoA$

If *reaction 21* proceeds more rapidly than *reaction 20*, the $E_{cl} - AcCoA$ formed in *reaction 23* will react with Ac*CoA which is present in excess, according to *reaction 21*. This will lead to a carboxyl labeled acetoacetyl derivative, in accordance with *reaction 24*:

24. $E_{cl} - AcCoA + Ac^*CoA \rightleftharpoons E_{cl} - AcAc^*CoA + CoA$

Thus, a high concentration of $AcAcO^-$ will favor the formation of unlabeled acetoacetyl CoA according to *reaction 22* and consequently asymmetry of label because of *23* and *24*. In the presence of an excess of Ac*CoA the dilution of labeled AcCoA by unlabeled AcCoA formed as a result of *21* and *22* would be negligible compared to the dilution of label in the $E_{cl} -$ Ac*CoA complex by the corresponding unlabeled complex. This situation will lead to a preferential labeling of the carboxyl group in acetoacetate. In fatty acid oxidation the unlabeled $E_{cl} -$ AcAc complex of *reaction 24* is provided by the $\omega - C_4$ fragment of the fatty acid chain, while the labeled Ac*CoA is formed by oxidation of the $-C^*O_2^-$.

[6] Unpublished observations of H. R. Mahler and D. S. Goldman.

[7] This combination of enzymes will be referred to below as the acetoacetate cleavage system (ACS). E_{cl} represents the cleavage site, and E_{Re} the replacement site of the ACS.

It has therefore been possible to duplicate with soluble enzymes, most of them highly purified, the oxidative conversions of fatty acids to acetoacetate or citrate, originally observed in mitochondria. Results leading to similar conclusions, although based on a different experimental approach, have also been reported at this symposium by Lynen.

The evidence just presented thus allows us to state that at least partial explanations have been provided by a reconstruction with purified, soluble enzymes for five of the key observations on mitochondrial fatty acid oxidation: the activation process, the non-accumulation of intermediates except acetoacetate, the oxidation of unsaturated, hydroxy and keto-acids, the exact process involved in β-oxidation, and the asymmetry of label in acetoacetate.

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DISCUSSION

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BY FOLLOWING the pyrophosphate liberation from ATP, rather than the hydroxamic acid formation, we have isolated fractions from hog liver which show a fatty acid activation effect that appears to be quite different from the ones reported by Dr. Mahler and others. It was found that four to twelve carbon fatty acid, ATP, and hydroxylamine reacted with a formation of sizeable amounts of pyrophosphate and relatively less hydroxamic acid without the addition of CoA. On purification, fractions were obtained which contained as little as 0.08 units of CoA per mg. of protein and in which adding catalytic amounts of CoA had little effect on the yield of pyrophosphate. However, if CoA is added in molecular proportion, hydroxylamine may be omitted and acyl CoA is formed in amounts comparable to the pyrophosphate.

As shown in table 1, in the CoA-free system both octanoate and hydroxylamine are necessary for the liberation of pyrophosphate. The role of hydroxylamine in this reaction is not well understood. The only compound which was found to replace hydroxylamine, in addition to CoA, was hydrazine. Nor do we have a ready explanation for the discrepancy between the pyrophosphate liberation and the hydroxamic acid formation. It may be mentioned, however, that acyl hydroxamic acid is not decomposed appreciably by the enzyme preparation. The question arises if there exist two different activation reactions, one with CoA as obligatory component, and another where presumably enzyme bound reactions with ATP and fatty acid occur preliminary to the transfer of the activated acyl group to CoA.

TABLE 1

Additions	17 minutes		45 minutes	
	P-P	Hydroxamic acid	P-P	Hydroxamic acid
	μM	μM	μM	μM
Octanoate	0.02	0	0	0.02
Hydroxylamine	0.02	0	0	0.05
Octanoate + hydroxylamine	0.80	0.14	1.39	0.21
Octanoate + CoA	0.76	0.53[1]	1.22	0.69[1]

System contains 0.55 mg enzyme, 5 μM ATP, 7 μM $MgCl_2$, 40 μM NaF, 10 μM GSH, 100 μM tris-(hydroxymethyl)aminomethane buffer pH 8.0; final volume 1.62 cc. Additions are 3μM octanoate, 200 μM hydroxylamine, and 310 units CoA. Incubated 1 hour at 37°.

[1] Hydroxylamine added after incubation to measure acyl CoA.

SUCCINYL COENZYME A AND ITS ROLE IN PHOSPHORYLATION[1]

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THE COUPLING of phosphorylation with the primary dehydrogenation of α-ketoglutarate to succinate and CO_2 has been well established by the work of Hunter, using tissue particle preparations (1). This reaction is of special interest because it is the only reaction of the Krebs cycle where substrate level phosphorylation occurs.

The use of complex, insoluble enzyme systems in previous studies prevented a detailed analysis of the reaction mechanism. In 1951, we reported on the preparation of a soluble enzyme system from pig heart capable of coupling the oxidative decarboxylation of α-ketoglutarate with the esterification of inorganic phosphate (2). In the preliminary work, α-ketoglutarate oxidation was measured as the dismutation of 2 molecules of α-ketoglutarate, in the presence of NH_3 to form succinate, CO_2 and glutamate. Just as in the case of pyruvate oxidation, it was found that the oxidation of α-ketoglutarate was dependent upon the presence of both DPN^+ and CoA (3, 4). The occurrence of the following reactions was postulated to explain these observations.[2]

Reaction 1 is similar to the pyruvate oxidation reaction where acetyl CoA is formed instead of the succinyl derivative. In order for pyruvate oxidation to proceed with catalytic amounts of CoA, the acetyl moiety of acetyl CoA must be transferred to acetyl acceptors such as orthophosphate or oxalacetate (3, 4). Since the oxidation of α-ketoglutarate in crude systems occurred in the absence of added acceptors, it was assumed that the heart fractions contained an enzyme catalyzing the hydrolysis of succinyl CoA (*reaction 2*). This enzyme was later isolated by Gergely *et al.* (5) and named succinyl CoA deacylase.

α-KETOGLUTARATE SYSTEM

(1) α-KETOGLUTARATE + CoA-SH + DPN^+ ⟶ SUCCINYL-S-CoA + CO_2 + DPNH + H^+ (DEHYDR.)

(2) SUCCINYL-S-CoA + H_2O ⟶ SUCCINATE + CoA-SH (DEACYLASE)

(3) Sum: α-KETOGLUTARATE + DPN^+ + H_2O $\xrightarrow{\text{(CoA-SH)}}$ SUCCINATE + CO_2 + DPNH + H^+

When the soluble pig heart preparations were supplemented with glucose and hexokinase, the dismutation of α-ketoglutarate was found to be coupled with the esterification of orthophosphate (2). The fact that this phosphorylation was not inhibited by iodoacetate or dinitrophenol, indicated its connection with the primary dehydrogenation of α-ketoglutarate. A clue to the mechanism of this coupled phosphorylation was given by the observation that the same enzyme preparations catalyzed the liberation of P from ATP if both CoA and succinate were present (2). Acetate could not replace succinate. These observations suggested that both the phosphorylation reaction and the dephosphorylation of ATP might be mediated by the overall reversible *reaction 4*.

(4) SUCCINYL-S-CoA + ADP + P ⟶ SUCCINATE + CoA-SH + ATP (P ENZYME)

(5) α-KETOGLUTARATE + DPN^+ + ADP + P $\xrightarrow{\text{(CoA-SH)}}$ SUCCINATE + CO_2 + DPNH + H^+ + ATP

The net result of the coupling of *reaction 1* with *reaction 4* would be *reaction 5*. The efficiency of the phosphorylation would depend on the competition between the utilization of succinyl CoA for ATP formation and succinyl CoA hydrolysis. The dephosphorylation of ATP in the presence of succinate and catalytic amounts

[1] The work described in this paper was supported by grants from the United States Public Health Service, the American Cancer Society (recommended by the Committee on Growth, National Research Council), and by a contract (N6onr279, T.O. 6) between the Office of Naval Research and New York University College of Medicine. The work was carried out in collaboration with Drs. C. Gilvarg, O. Cori, and S. Ochoa.

[2] The following abbreviations are used: DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN^+, triphosphopyridine nucleotide; CoA or CoA-SH, coenzyme A (reduced); succinyl CoA or succinyl-S-CoA, succinyl coenzyme A; acetyl CoA or acetyl-S-CoA, acetyl coenzyme A; GSH, reduced glutathione; ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosine-5′-phosphate (muscle adenylic acid); PP, pyrophosphate; P, orthophosphate; TRIS, tris(hydroxymethyl)aminomethane.

of CoA was visualized as the coupling of *reaction 4* (right to left) with *reaction 2*. As will be seen later, this assumption proved to be correct.

In order to obtain direct evidence for the reaction sequence just outlined, the crude enzyme system was subjected to fractionation in an attempt to separate the individual enzymes or groups of enzymes involved. These enzymes will be referred to for convenience as α-ketoglutaric dehydrogenase (dehydrogenase), succinyl CoA deacylase (deacylase), and phosphorylating enzyme (P enzyme), respectively, but it remains an open question whether the dehydrogenase and P enzyme are single entitites. The dehydrogenase and deacylase have been purified and studied independently in Green's laboratory (5, 6). More recently Littlefield and Sanadi have also purified and studied the P enzyme (7).

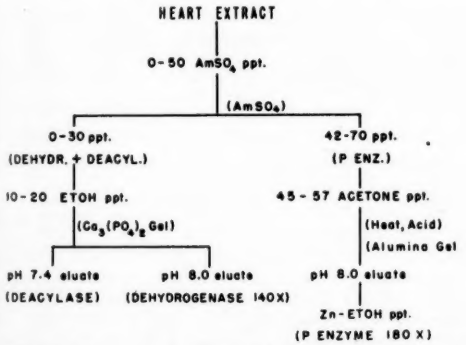

Fig. 1. FRACTIONATION of heart α-ketoglutarate system.

An outline of our purification procedure is given in figure 1. The dehydrogenase was assayed either by measuring the rate of CO_2 evolution from α-ketoglutarate in the dismutation system, or spectrophotometrically by measuring the rate of DPN^+ reduction in the presence of catalytic amounts of CoA and excess deacylase. The P enzyme was routinely assayed by measuring the rate of succinohydroxamic acid formation in the presence of ATP, catalytic amounts of CoA and an excess of hydroxylamine, or spectrophotometrically, by measuring the rate of DPN^+ reduction in the presence of ADP, orthophosphate and excess dehydrogenase (8, 9). Our starting material for all these enzymes was a fraction obtained from 0.2 M KCl extracts of washed minced pig heart by precipitation with ammonium sulfate at 50% saturation (0–50 fraction). On refractionation of this material with $AmSO_4$, the 0–30 fraction contained the dehydrogenase and deacylase, while the 42–70 fraction contained the P enzyme. The dehydrogenase and deacylase were separated from each other by adsorption on calcium phosphate gel and fractional elution with phosphate buffer. The P enzyme was further purified as shown. The final preparation of dehydrogenase represents

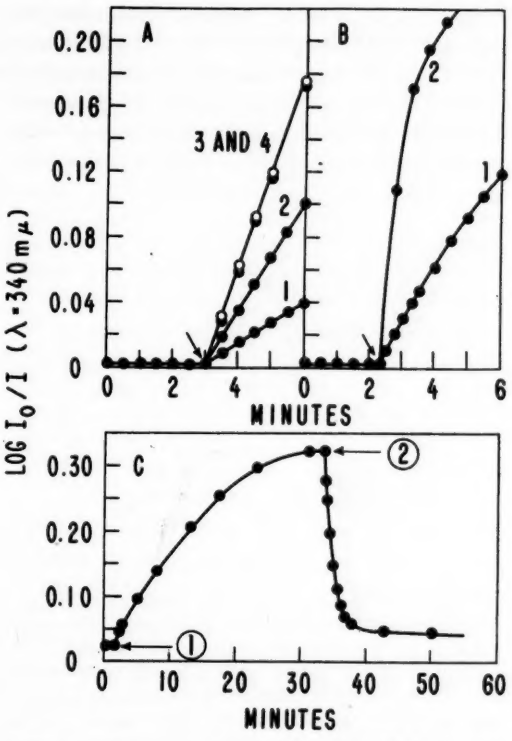

Fig. 2. OPTICAL TESTS with α-ketoglutaric dehydrogenase; effect of CoA and deacylase. The system contained in all cases, potassium phosphate buffer, pH 7.4, cysteine, α-ketoglutarate, DPN^+, CoA, and purified α-ketoglutaric dehydrogenase. Final volume made up with water to 3.0 ml. In *A*, the amounts of dehydrogenase were: *curve 1*, 0.017 mg; *curve 2*, 0.043 mg; *curves 3* and *4*, 0.086 mg protein. In *B*, the amounts of dehydrogenase were: *curve 1*, 0.017 mg; *curve 2*, 0.064 mg protein. In *C*, 0.172 mg dehydrogenase was used.

a 140 fold purification over the initial extracts, that of the P enzyme about 180 fold purification.

Typical experiments with the purified enzymes are illustrated in figure 2 (10). In these experiments, the reactions were followed spectrophotometrically by measuring the rate of DPNH formation at 340 mμ. Figure 2*A* presents evidence for the coupling of dehydrogenase and deacylase. The various curves were obtained with different amounts of dehydrogenase, constant deacylase,

and catalytic amounts of CoA. The last addition (indicated by the arrow) was dehydrogenase in *curves 1* and *2*, deacylase in *curve 3* and CoA, which had first been incubated with cysteine, in *curve 4*.

Figure 2*B* shows results obtained with the dehydrogenase with stoichiometric amounts of DPN^+ and CoA but in the absence of deacylase. *Curves 1* and *2* were obtained with different amounts of dehydrogenase. The last addition in *curve 1* was CoA and in *curve 2* was dehydrogenase. These experiments serve to emphasize the point that deacylase is required only with catalytic amounts of CoA. With stoichiometric

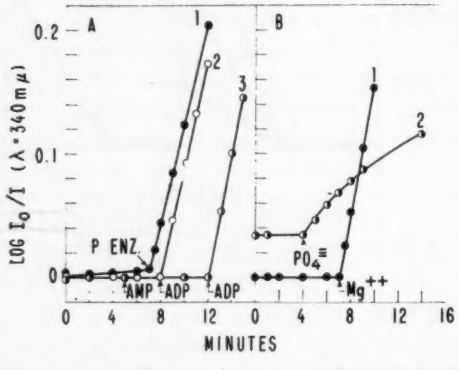

Fig. 3. Optical test with α-ketoglutaric dehydrogenase; coupling with P enzyme. The complete system contained, besides the additions as indicated, TRIS buffer, pH 7.4, catalytic amounts of CoA and DPN, cysteine, α-ketoglutarate, and purified α-ketoglutarate dehydrogenase. In *A*, the amount of α-ketoglutaric dehydrogenase was 0.086 mg, and of purified P enzyme, 0.59 mg protein. In *B*, the amount of α-ketoglutaric dehydrogenase was 0.061 mg, and 0.52 mg P enzyme protein.

amounts of CoA, the reaction proceeds rapidly in the absence of deacylase. The formation of succinyl CoA in the latter type of experiment was shown by the disappearance of SH and its re-appearance on addition of deacylase. Succinyl CoA could be isolated from the reaction mixture by adsorption on charcoal and elution with aqueous pyridine. Similar results have been obtained by Green and his collaborators (6). *Curve C* shows the dehydrogenase reaction with catalytic amounts of CoA, deacylase and limiting amounts of α-ketoglutarate added at *arrow 1*. At *arrow 2*, 10 μM of pyruvate was added which caused the oxidation of DPNH through lactic dehydrogenase present in the deacylase preparation. This experiment shows that DPNH was formed by the oxidation of α-ketoglutarate catalyzed by the dehydrogenase.

The coupling of dehydrogenase and P enzyme is shown in figure 3. The complete system contained, in addition to the enzymes, a catalytic amount of CoA, DPN^+, PO_4, ADP and Mg^{++}. *Curves 1A* and *2A* show that there is no reaction until either P enzyme or ADP is added. *Curve 3A* shows that AMP cannot replace ADP.

Table 1. *A.* Role of P enzyme in phosphorylation (*values in micromoles*)

Dismutation (60 min., 25°)	$\Delta\alpha$-Keto-glutarate	ΔCO_2	ΔP	$\Delta P/\Delta CO_2$
Dehydrogenase	−4.7	+2.7	0	0
Dehydrogenase + P enzyme	−8.7	+3.6	−2.7	0.75

B. P enzyme reaction (*values in micromoles*)

	Δ Hydroxamic	ΔSH	ΔCoA
Succinate + ATP + CoA (30 min., 37°)	+0.62	−0.63	−0.70
	ΔSH	ΔP	ΔGlucose-6-P
Succinyl CoA + ADP + P (Glucose + hexokinase, 20 min., 20°)	+1.11	−0.84	+0.78

The complete system for the dismutation experiment contained $MgCl_2$, tris buffer, pH 7.4, DPN^+, CoA, NH_4Cl, glucose, KF, ATP, cysteine, orthophosphate, and 50 μM of α-ketoglutarate. Besides the enzymes indicated, glutamic dehydrogenase and hexokinase were present in excess. For the succinate, ATP, CoA reaction, the system contained succinate, tris buffer, pH 7.4, $MgCl_2$, ATP and 2.8 μM of CoA, which was 60% reduced, and purified P enzyme. For the succinyl CoA, ADP, P experiment, the system contained tris buffer, pH 7.4, $MgCl_2$, orthophosphate, glucose, hexokinase, 2.7 μM of succinyl CoA and purified P enzyme.

The requirement for Mg^{++} and orthophosphate is shown by *curves 1B* and *2B*. Prolonged dialysis of the enzymes in the latter case, in order to remove phosphate, resulted in their partial inactivation.

The P enzyme-dependent esterification of inorganic phosphate during α-ketoglutarate oxidation is shown in the upper part of table 1. In this experiment, α-ketoglutarate was oxidized through dismutation as previously outlined, in the presence of NH_3, dehydrogenase, crystalline glutamic dehydrogenase and catalytic amounts of DPN^+ and CoA. ATP, glucose and

hexokinase were also present. That the reaction proceeded at all in the absence of P enzyme was due to contamination of the dehydrogenase with traces of deacylase.

The lower part of table 1 illustrates the stoichiometry of the reversible reaction catalyzed by the P enzyme. The first experiment shows the formation of succinyl CoA from succinate, ATP and CoA as evidenced by the appearance of hydroxamic acid and the disappearance of equivalent amounts of both SH and CoA (the latter determined with transacetylase by Stadtman's (11) arsenolysis reaction). The second experiment shows that cleavage of succinyl CoA (appearance of SH), in the presence of orthophosphate, ADP, hexokinase and glucose is accompanied by the esterification of a stoichiometric amount of phosphate and the formation of an equivalent amount of glucose-6-phosphate. The succinyl CoA was prepared synthetically by the method of Simon and Shemin (12). Although the equilibrium constant of the P enzyme reaction has not been determined, the ease of reversibility indicates that the free energy content of the thioester linkage of succinyl CoA is not too different from the pyrophosphate bond of ATP.

The requirements for the reaction between succinate, ATP and CoA, as catalyzed by the P enzyme when CoA is present in catalytic amounts, are shown in table 2. In the presence of hydroxylamine, the formation of succinohydroxamic acid was accompanied by the liberation of an equivalent amount of orthophosphate thus approaching the theoretical phosphate-hydroxamic acid ratio of 1. The small excess of phosphate liberated over hydroxamic acid formed was probably due to the slow non-enzymatic hydrolysis of succinyl CoA during the incubation period. There was no reaction in the absence of either succinate, ATP, CoA or Mg^{++} and, although not shown in the table, acetate could not replace succinate. In the absence of hydroxylamine, no orthophosphate was liberated unless deacylase was present, as shown in the lower half of the table.

The reaction between succinate, ATP and CoA is not only catalyzed by an enzyme different from that concerned with the similar reaction between acetate, ATP and CoA, but its mechanism is different. As recently shown by Lipmann and coworkers (13), the acetate reaction results in the reversible formation of acetyl CoA, AMP and inorganic pyrophosphate, while, as we have seen, the succinate reaction results in the reversible formation of succinyl CoA, ADP and orthophosphate.

Although the overall mechanism of the phosphorylation coupled with α-ketoglutarate oxidation has now been elucidated, the intimate mechanism of the reaction catalyzed by the P enzyme remains obscure. Lynen has postulated CoA-S-phosphate as an intermediate (14). This would be formed by reaction between ATP and CoA, or by phosphorolysis of the acyl CoA in the reverse direction. So far we have not succeeded in establishing the formation of an intermediate product in the succinate-ATP-CoA reaction or in resolving the P enzyme into distinct enzymatic components.

TABLE 2. COMPONENTS OF P ENZYME SYSTEM WITH CATALYTIC CoA (*incubation time, 30 minutes. Values in micromoles*)

System	Temp.	ΔP	ΔHydroxamic	ΔP/ΔHydroxamic
Complete (hydroxylamine)	37°	+2.1	+1.8	1.16
Complete (hydroxylamine)	37°		+2.76	
No Succinate			+0.10	
No ATP			+0.14	
No CoA			+0.10	
No Mg^{++}			+0.14	
Complete (no hydroxylamine)	25°	+0.06		
Complete + deacylase		+2.74		

The complete system contained, besides the enzymes as indicated, tris buffer, pH 7.4, succinate, ATP, CoA, GSH, $MgCl_2$ and a large excess of hydroxylamine. In the last experiment, purified succinyl CoA deacylase was added instead of the hydroxylamine.

From recent experiments carried out in our laboratory, it would appear that a free intermediate in the reaction is unlikely. There was no detectable reaction when ATP and CoA were incubated together in the presence of the purified P enzyme, as measured by SH and CoA determinations (15). The possibility was considered that such a reaction might not proceed to any measurable extent because of a very unfavorable equilibrium. In an attempt to overcome such a high free energy barrier, the P enzyme was coupled with the phospho-enol-pyruvate-ADP system in the presence of the purified kinase. In this case, a reaction between CoA and ATP

would be detectable by measuring the liberation of pyruvate with DPNH and lactic dehydrogenase spectrophotometrically. Even with such a sensitive test, no reaction was discernible (15). Tests carried out with succinyl monophosphate indicate that this compound does not participate in the reaction as a free intermediate (15). Although there is now evidence that both CoA-P and succinyl phosphate are not free intermediates, the possibility still exists that either compound could be involved as an enzyme-bound intermediate.

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DISCUSSION

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We wish to report on estimations of the equilibrium constant of the adenosine triphosphate (ATP)-coenzyme A (CoA) - acetate reaction:

$$\text{ATP} + \text{CoA} + \text{acetate} \rightleftharpoons \text{acetyl CoA} + \text{AMP} + \text{pyrophosphate}$$

We followed the reaction spectrophotometrically (1). The changes in light absorption at 232 mμ were observed and are due to the formation or loss of a thiol-ester bond, in this instance, the thiol-ester bond of acetyl CoA. A typical experiment is shown in figure 1. When ATP, CoA and acetate are mixed with yeast enzyme (2). equivalent amounts of acetyl CoA, adenosine monophosphate (AMP) and pyrophosphate are formed and the absorption reaches a steady value in about 10 minutes. The reaction is reversed when an excess of pyrophosphate is added; this causes a rather rapid decrease in the acyl-mercaptan absorption, and the extent of the decrease is related to the pyrophosphate concentration. If the same amount of pyrophosphate is initially added, the rate of acetyl CoA formation is considerably slower. However, the maximum for this curve approaches the absorption observed in the reversal experiment. With higher pyrophosphate concentrations, a secondary decline eventually occurs, which is not understood at the present time.

The value for the equilibrium constant ranges from 1.4 to 5.4 with 2.7 as an average value (table 1). This value indicates, in confirmation of the data of Stadtman (3) and Stern *et al.* (4), that the energy of the phosphoryl bonds of ATP and the acyl-mercapto bond of acetyl CoA are nearly equivalent. In addition it appears that the energy of the second pyrophosphate bond of ATP is equivalent to the terminal pyrophosphate bond.

TABLE 1. EQUILIBRIUM CONSTANT FOR ATP-CoA-ACETATE REACTION

Experiment	Final concentration						K_1
	ATP	CoA	Acetate	Acetyl CoA	AMP	Pyrophosphate	
	$\mu M/ml.$	$\mu M/ml.$	$\mu M/ml.$	$\mu M/ml.$	$\mu M/ml.$		
1	5.5	0.2	5.5	2.3	2.5	2.5	2.4
2	0.127	0.007	0.127	0.073	0.073	0.073	3.4
3	0.078	0.068	1.82	0.092	0.092	2.89	2.6
4	0.10	0.09	1.87	0.059	0.059	8.06	1.7
5	0.09	0.072	1.86	0.071	0.071	8.07	3.3
6	0.177	0.028	0.65	0.072	0.23	1.07	5.4
7	0.168	0.019	3.35	0.081	0.24	1.08	1.9
8	0.105	0.059	1.68	0.066	0.066	8.72	3.6
9	0.117	0.071	1.70	0.054	0.054	7.9	1.6
10	0.280	0.054	1.69	0.062	0.062	9.3	1.4
						Average =	2.7

[1] $$K = \frac{(Acetyl\ CoA)\ (AMP)\ (Pyrophosphate)}{(Acetate)\ \ (AMP)\ \ \ (CoA)}$$

In *Exper. 1* the reaction vessel contained in 1 ml: 8 μM ATP; 2.5 μM CoA; 40 μM KF; 8 μM $MgCl_2$; 20 μM H_2S; 160 μM tris(hydroxymethyl)-aminomethane buffer, pH 7.5, and 0.02 ml yeast enzyme (20 U). The vessel was incubated at 37° for 30 min. when the amount of acetyl CoA and pyrophosphate were measured by the hydroxamic acid method (6) and by a colorimetric method[2], respectively. *Expers. 2* through *10* were carried out at room temperature in the Beckman spectrophotometer like the experiments illustrated in figure 1. The acetyl CoA concentration was determined from the final absorption at 232 mμ, using the value 4.1×10^6 cm.^{2}/m as the extinction coefficient for acetyl CoA. The pyrophosphate present was determined colorimetrically[2] but the amounts of ATP, CoA, acetate and AMP were determined by difference.

Early experiments seemed to support the hypothesis that a reaction occurs between pyrophosphate and acetyl CoA, presumably with the formation of CoA-pyrophosphate (2). However, in more recent experiments with purer enzyme and acetyl CoA, pyrophosphate, without the addition of adenosine monophosphate, does not cause a loss of acetyl CoA in the presence of the enzyme. The earlier results presumably were due to the presence of small amounts of AMP as impurities.

Furthermore, attempts to realize a separate reaction between ATP and sulfhydryl-CoA gave essentially negative results. In this type of experiment, it is important to realize the difficulties of excluding the presence of small amounts of acetate.

[2] Inorganic pyrophosphate gives a slowly developing color (7) with the Fiske and Subbarow molybdate reagents. This color increase has been utilized to develop a convenient colorimetric method for the determination of inorganic pyrophosphate, which will be described elsewhere.

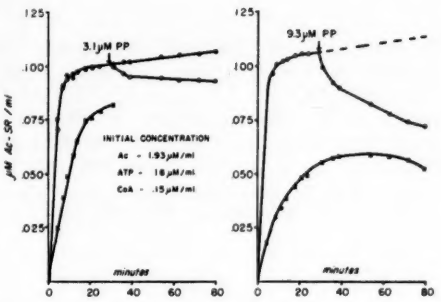

Fig. 1. REVERSIBILITY of ATP-CoA-acetate reaction by addition of pyrophosphate. Each cell contained in 1 ml: 0.15 M CoA·SH (reduced with potassium borohydride (5)); 0.16 μM ATP; 1.93 μM potassium acetate; 35 μM KF; 2 μM $MgCl_2$; 40 μM tris(hydromethyl)aminomethane buffer, pH 7.5; and 0.001 ml (1 unit) of yeast enzyme fraction 4 (5).

Left: ● = control cell to which no pyrophosphate is added. ○ = cell which contains the same reactants as the control tube except that 3.1 μM potassium pyrophosphate buffer, pH 7.5, are added at the time indicated by the arrow. X = cells which contain the same reactants as the control cell except that 3.1 μM potassium pyrophosphate buffer, pH 7.5, are added at the start. *Right:* Curves as above, except that 9.3 μM of the pyrophosphate buffer were added to the ○ and X curves rather than 3.1 μM.

ADDENDUM

Recent tests by Jones *et al.* (8) with radioactive acetate and pyrophosphate suggest the following mechanism for the ATP-CoA-acetate reaction[3]:

(1) $Ex + Ad - P \sim PP \rightleftharpoons Ex \sim P - Ad + PP$

(2) $Ex \sim P - Ad + HSCoA \rightleftharpoons Ex \sim SCoA + AdP$

(3) $Ex \sim SCoA + CH_3COOH \rightleftharpoons Ex + CH_3CO \sim SCoA$

The initial reaction of ATP with the enzyme to yield AMP-enzyme with elimination of ATP is indicated by a rapid exchange of pyrophosphate and ATP in absence of CoA or other components.

A pronounced inhibition by CoA of the ATP-PP exchange indicates *reaction 2*; CoA competes with AMP for the enzyme and therefore decreases the concentration of E-PAd, a component of *reaction 1*. Finally, *reaction 3* is indicated by an exchange of acetate with acetyl CoA in the absence of all other components.

[3] The following abbreviations are used in these equations: E = enzyme, AdP ~ PP = ATP, PP = pyrophosphate.

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METABOLISM OF THIOLESTERS OF GLUTATHIONE[1]

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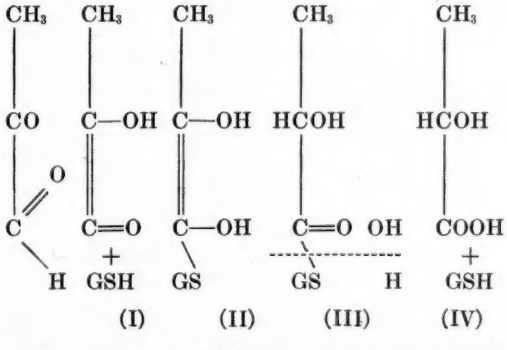
THIS AFTERNOON we have received a very clear picture of the manifold varieties of coenzyme A activity. There emerge two major functions of this coenzyme, one of which is concerned with the transfer of acyl groups, the other with the oxidation of aldehydes and keto acids- coupled to 'substrate level' phosphorylation.

Our own work has been concerned with another SH compound that is widely distributed in cells: glutathione. We find that thiolesters of this cofactor also participate in oxidation-reduction, as well as in acyl group transfer reactions. In addition to these two types of reaction, we shall present some evidence for a third function, namely that of a carrier facilitating the oxidation of certain two-carbon compounds.

We became interested in glutathione by a curious pathway. In the course of studies on brain metabolism, we made some observations which led us to suspect that glutathione may participate in the oxidation of aldehydes catalyzed by glyceraldehyde-3-phosphate dehydrogenase. We attempted to demonstrate the presence of glutathione in this enzyme and required for this purpose a sensitive assay for glutathione. It was known that the conversion of methylglyoxal to lactic acid, when catalyzed by glyoxalase, requires glutathione; a manometric method for glutathione assay based on this reaction had been described by Woodward (1). For our purpose however, this method was not sufficiently sensitive and, having suffered from a Warburg nystagmus in the early days of our scientific childhood, we have turned in recent years to the more sensitive spectrophotometric methods. This indeed has earned us the title of being Beckman cellular physiologists.

Since we expected a decrease in light absorption in the ultraviolet when methylglyoxal is converted to lactic acid we were surprised to find that, when a dilute yeast extract (glyoxalase I) was added to glutathione and methylglyoxal, there was an increase in the absorption at 240 mμ as shown in figure 1. The increment in light absorption indicated the formation of an intermediate, which was then found to be broken down by a second enzyme (glyoxalase II) to lactic acid and glutathione. The intermediate was isolated as a barium salt and found to be the thiolester lactylglutathione (2). These findings together with the work of others, particularly Yamazoye (3), Hopkins and Morgan (4), Crook and Law (5), have led us to a formulation of a hypothesis shown below.



It should be made clear that of the intermediates suggested in this scheme only lactylglutathione has been shown to accumulate. Recently Wieland and Köppe (6) have prepared synthetic DL-lactylglutathione and found it to be hydrolyzed by a crude liver fraction which contained glyoxalase II. Dr. Wieland was kind enough to send us some of the synthetic substrate and we were able to confirm his findings with a purified preparation of glyoxalase II which hydrolyzed the synthetic thiolester at about the same rate as the natural thiolester. It was of interest to note that the hydrolysis of the synthetic preparation also went to completion despite the fact that it contained both D- and L-lactylglutathione.

The other intermediates pictured in the scheme are hypothetical. To justify the postulated first reaction in which the ketene form of methylglyoxal interacts with the SH group of gluta-

[1] This investigation was supported by a grant from the United States Public Health Service.

thione, we have looked for refuge on the surface of the enzyme, since in solution the ketene would have a very short life expectancy. A more likely intermediate to visualize is the thiohemiacetal between the SH and the aldehyde group. This indeed had been suggested many years ago by Jowett and Quastel (7) and more recently by Wieland and Köppe (6). But it should be pointed out that the simple chemical addition product, which had been prepared by Schubert (8) was ruled out as the intermediate by Yamazoye (3) as well as by our own observations. However, I should like to draw attention to the fact that the structure of the chemical addition product has never been established and a reaction between the keto group of methylglyoxal and the SH group has not been excluded.

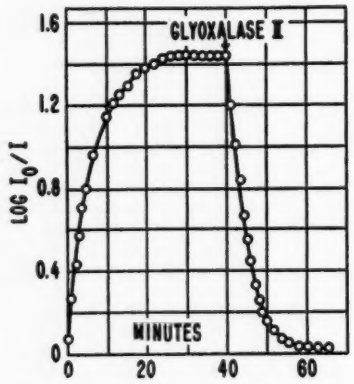


Fig. 1. SPECTROPHOTOMETRIC demonstration of glyoxalase I and glyoxalase II.

Before leaving this subject matter I should like to mention a simple method of purifying the thiolesters of glutathione with Dowex-50 (2% cross-linkage) by a procedure described by Dowmont and Fruton (9) for the isolation of peptides (fig. 2).

Stimulated by these findings with glyoxalases, we proposed a similar mechanism for the oxidation of other aldehydes such as glyceraldehyde-3-phosphate. This mechanism included a phosphorolytic cleavage of the thiolester of glutathione with preservation of energy. The experimental evidence obtained in our laboratory for the two step mechanism of triose phosphate oxidation has been published (10) and need not be repeated. The discussion by Miss Harting will present some of her interesting work on the transfer reactions to coenzyme A catalyzed by triose phosphate dehydrogenase. Our own work was more concerned with acyl group transfer to added glutathione as well as to glutathione linked to the enzyme. We have demonstrated the existence of an acyl-enzyme and were able to precipitate the acyl enzyme from solution. After proteolytic digestion a thiolester, now under investigation, is liberated from the denatured protein.

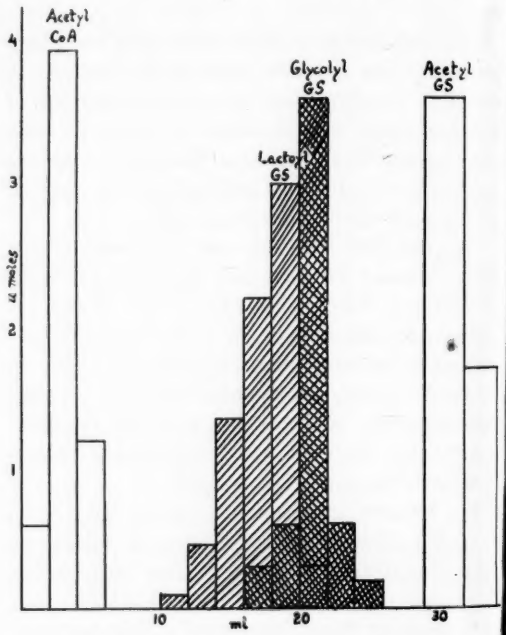


Fig. 2. PURIFICATION of thiolesters on Dowex 50. Column 20 x 1.0 cm. Formic-ammonium formate buffer 0.2 M pH 3.08. Flow rate 4.2 ml/hour.

We can see that there exist some striking analogies between coenzyme A and glutathione, but there appears to be considerable specificity in the choice of a SH coenzyme by the enzyme systems. Thus, the glyoxalases which react with glutathione do not react with coenzyme A, and the citrate forming condensing enzyme cannot utilize acetylglutathione in place of acetyl CoA.

We have recently encountered an enzymatic reaction which establishes a relationship between these two SH cofactors and also links them to other oxidation reduction systems of the cells. This reaction which involves hydrogen transfer from an SH compound to an SS compound is catalyzed by enzymes that are widely distributed. One of these enzymes has been partially purified from liver and studied in greater detail and was found to catalyze the reaction:

reduced glutathione + homocystine $\rightleftharpoons$
oxidized glutathione + homocysteine

This reaction was usually performed at concentrations of glutathione at which the rate of the nonenzymatic transfer was negligible. In order to accomplish this, small amounts of reduced glutathione were continuously regenerated by the addition of a reducing system consisting of alcohol, alcohol dehydrogenase, DPN and glutathione reductase. As will be shown elsewhere, glutathione reductase from either yeast or liver catalyzes the hydrogen transfer not only from reduced TPN but also from reduced DPN, though at a slower rate with the latter. The formation of homocysteine in this system was measured by the nitroprusside test, correcting for the small amounts of reduced glutathione present. Homocysteine was also demonstrated by forming the lactone in the presence of strong acids. With partially purified preparations of the hydrogen transferase, reduction of homocystine was dependent on the presence of glutathione, glutathione reductase and the transferase, thus demonstrating that the nonenzymatic transfer was negligible under the conditions of the test.

TABLE 1. CO_2 EVOLUTION IN THE PRESENCE OF FORMALDEHYDE AND GLYOXAL IN YEAST EXTRACTS

Addition to yeast extracts[1]	μM CO_2 in 20 min.
Formaldehyde (40μ moles) + DPN (1μ mole)	5.1
Formaldehyde	1.8
DPN	<0.3
Formate + DPN	<0.3
Formate + DPN + ATP (5μ moles)	<0.3
Glyoxal + DPN	12.5

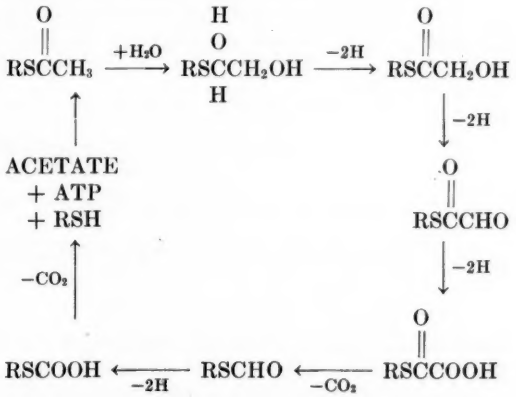
[1] 1.5 ml of a crude extract from dried yeast was used which contained only small amounts of DPN. The reaction was carried out in a final volume of 2 ml in a Warburg vessel at 30° in an atmosphere of nitrogen.

Crude liver preparations to which alcohol, alcohol dehydrogenase and DPN were added were found to catalyze the reduction of a wide variety of S–S compounds including coenzyme A, tetraethyl-thiuram disulfide, lipoic acid and even insulin according to the following reaction:

$$\text{Reduced DPN} + \text{RSSR} \rightarrow \text{oxidized DPN} + 2\text{RSH}$$

Finally, I should like to turn to a third function of glutathione. We have reported that yeast extracts contain a DPN linked thiolester dehydrogenase which catalyzes the oxidation of lactylglutathione (2) as well as glycolylglutathione (11). On the other hand these thiolesters can be reduced by DPNH and another enzyme (thiolester reductase) present in yeast. In crude extracts from dried yeast where both these enzymes are present, a DPN linked dismutation occurs in the presence of thiolesters. With either glyoxal or glycolylglutathione as substrate a rapid evolution of CO_2 can also be demonstrated.

Based on studies with isotopes, H. A. Krebs (12) has concluded that the Krebs cycle is not an important pathway as far as acetate oxidation in yeast is concerned and he suggested a direct oxidation of acetyl CoA as an alternative pathway. I have ventured to present below what we might call a new Krebs Cycle.



Of the reactions postulated, there is good evidence for the first reaction in which acetyl CoA is formed from ATP, acetate and CoA (13). The next step, which we visualize as an uptake of water by the enol form of acetyl CoA or acetyl glutathione, is hypothetical and no direct evidence is available for such a reaction. The evidence for the remaining steps of this cycle can be summarized as follows: Dehydrogenases and reductases for thiolesters, such as glycolylglutathione, have been demonstrated and partially purified from yeast. Decarboxylation of the thiolesters was shown by CO_2 evolution. The last steps of oxidation of a one carbon unit are again hypothetical. However, we have recently found a reaction to occur in yeast extracts with formaldehyde as substrate. The addition of the formaldehyde and DPN leads to evolution of CO_2, while formic acid with or without ATP is inactive as shown in table 1.

In closing, I would like to point out that this cycle has a number of attractive features. Since the oxidation steps of both the two carbon and one carbon units have been shown to be DPN linked, a utilization of the energy of oxidation

can be expected by the process of coupled phosphorylation. Moreover, a thiolester such as glyoxylglutathione may participate in transamination reactions which would lead to the formation of glycylglutathione, a possible intermediate in peptide formation.

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DISCUSSION

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Glyceraldehyde-3-phosphate dehydrogenase has been shown to have a broad specificity and to catalyze the oxidation of a number of aldehydes (1, 2). In connection with this symposium the oxidation of acetaldehyde to acetyl phosphate (*reaction 1*) is of rather special interest.

(1) acetaldehyde + phosphate + DPN ⇄ acetyl phosphate + $DPNH_2$

The oxidation product, acetyl phosphate, can act as substrate for a second type of reaction, namely, a transacetylation which is also catalyzed by the triose phosphate dehydrogenase (3). In the second reaction (*reaction 2*) the acetyl group of acetyl phosphate is transferred to CoA and yields acetyl CoA.

(2) acetyl phosphate + CoA ⇄ acetyl CoA + phosphate

These two reactions, the production of acetyl phosphate and the transacetylation to CoA, can be catalyzed by the crystalline dehydrogenase isolated from either rabbit muscle or yeast. Similar reactions have been observed in bacteria (4, 5), but neither of these activities had previously been found for enzymes from mammalian tissues or from yeast. Although the biological significance of these catalyses in mammalian metabolism is still problematical, it is nevertheless interesting that a single enzyme can catalyze not only the formation of acetyl phosphate but also the transfer of this acetyl group to CoA as acceptor.

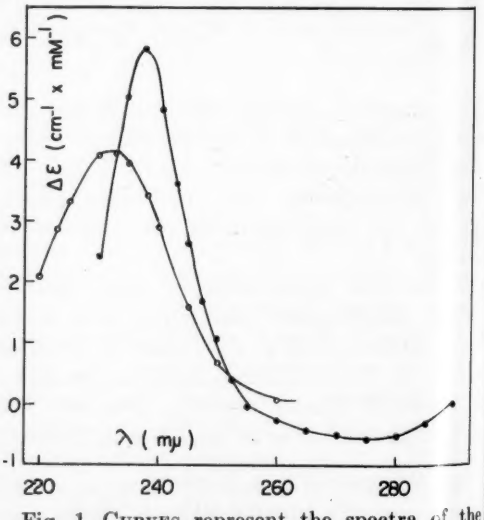

Fig. 1. Curves represent the spectra of the acetyl enzyme complex —●—●— and acetyl glutathione —○—○—.

In transacetylase reactions involving acetyl

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phosphate, it is generally assumed that acetyl phosphate first forms an acetyl enzyme complex, and the acetyl group is subsequently transferred to another acceptor, such as CoA (5). The exchange of acceptors may be schematically represented by the following equation:

$$\text{(3) acetyl phosphate} + \text{enzyme} \overset{\pm \text{ phosphate}}{\rightleftharpoons} \text{acetyl enzyme} \overset{\pm \text{ CoA}}{\rightleftharpoons} \text{acetyl CoA} + \text{enzyme}$$

The three criteria for testing such a hypothesis, as established by Hassid, Doudoroff, and Barker (6), are: *1*) exchange of the phosphate of acetyl phosphate with radioactive inorganic phosphate, *2*) arsenolysis of acetyl phosphate, *3*) transfer of the acetyl group to still other acceptors. These three criteria have been fulfilled in the case of the triose phosphate dehydrogenase (3, 7); therefore, the existence of an acetyl enzyme complex was suggested in the transfer reactions.

It was then decided to attempt a direct spectroscopic identification of the acetyl enzyme intermediate. Since Dr. Racker has focused our attention on the role of the thiol group in this reaction (7), we have investigated the spectra in the ultraviolet region where the known thiol esters have a characteristic absorption peak. No previous spectroscopic identification of compounds of a dehydrogenase and its substrate has been described because the absorption of the protein itself in the ultraviolet is so large that a special spectrophotometric system is required (8).

Upon the addition of acetyl phosphate to triose phosphate dehydrogenase, the formation of an acetyl enzyme complex can be observed. When the spectrum of the acetyl enzyme compound (fig. 1) is compared to that of a typical thiol ester, acetyl glutathione, it may be seen that the curves are not the same. This fact suggests that if the acetyl group is linked to a thiol group on the enzyme it does not have the same properties as a simple thiol ester, or that the linkage to the protein is of some other type. Whatever the nature of the bond, it is clearly of an energy rich type in view of the readily reversible transfer of the acetyl group between compounds such as acetyl phosphate and acetyl CoA.

The acetyl enzyme compound is decomposed by arsenate. This would be expected since the enzyme has been shown to catalyze the arsenolysis of acetyl phosphate. In accordance with the previous scheme, this reaction may be written.

$$\text{(4) acetyl phosphate} + \text{enzyme} \overset{\pm \text{ phosphate}}{\rightleftharpoons} \text{acetyl enzyme} \overset{\pm \text{ arsenate}}{\rightleftharpoons} \text{enzyme} + \text{(acetyl arsenate)} \longrightarrow \text{acetate} + \text{arsenate}$$

The arsenolysis of the enzyme substrate complex supports the view that this complex may be the active intermediate in the transfer reactions.

In summary, an acetyl enzyme compound of triose phosphate dehydrogenase has been identified spectroscopically. With the further identification of enzyme substrate complexes with the dehydrogenases, perhaps a more detailed study of the mechanism of oxidation will be possible.

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AMERICAN INSTITUTE OF NUTRITION

Symposium on

Metabolic Significance of B-Vitamins

Chairman: ESMOND E. SNELL

Summary of metabolic functions of niacin, riboflavin and vitamin B_6. ESMOND E. SNELL. *Department of Chemistry, University of Texas, Austin*

Metabolic functions of pantothenic acid. G. DAVID NOVELLI. *Massachusetts General Hospital and Harvard Medical School, Boston*

Metabolic functions of thiamin and lipoic acid. LESTER J. REED. *Department of Chemistry, University of Texas, Austin*

Metabolic functions of biotin. HENRY A. LARDY. *Department of Biochemistry, University of Wisconsin, Madison*

The four papers of this Symposium will be published in PHYSIOLOGICAL REVIEWS, *Volume 33, pages 509–565, October 1953*

THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS

Symposium on Immunochemical Analysis of Systems Containing Multiple Antigens[1]

Chairman: A. M. PAPPENHEIMER, JR.

ANTIGEN-ANTIBODY REACTIONS IN GELS

ELMER L. BECKER

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In 1946 Oudin first pointed out the significance and the importance of the antigen-antibody reactions occurring in gels though observations of such reactions had been made many times before. Since that date a great many different techniques and refinements of techniques have been elaborated for carrying out such reactions. These various techniques have been applied to the immunochemical study and fractionation of the proteins of serums, of the antigens in ragweed extracts, of diphtheria and scarlet fever toxins, the developing frog embryo and in a number of bacteriological problems; a start has been made in their application to viruses and Dr. Telfer later in the program will give an illustration of the application of an antigen-antibody gel technique to problems of insect metamorphoses. We have shown how under carefully chosen conditions one can use the Oudin technique for the determination of the diffusion coefficients of antigens. This is far from a complete list but indicates the widespread usefulness of the various techniques.

There are two general types of methods for carrying out antigen-antibody reactions in gels. Using the terminology introduced by Oudin, the first is termed 'simple' diffusion, in which one of the reactants, usually antigen, diffuses into a gel containing a constant concentration of the other reactant, usually antibody. An example of this is the technique of Oudin and a modification by Surgalla which will be described in more detail later. The second general class of techniques is that of 'double diffusion'. Here, the two reactants diffuse into the gel which originally contained neither. The techniques of Ouchterlony, Elek, Pope and Bowen are all varieties of the latter type.

A very excellent, detailed review by Oudin of the theory and technique of the various gel techniques and the results to be obtained is contained in vol. 5 of *Methods in Medical Research*. I shall therefore concentrate my attention only on certain points and because of limitations in time shall restrict my discussion to the technique of Oudin.

In this technique the antiserum is mixed with warm liquid agar and the mixture introduced into a thin bore glass tube. The tube is cooled and the antigen solution is carefully overlayed on the now solid agar. An antigen antibody precipitate is formed as a band which progresses down the tube as more and more antigen diffuses into the agar. A single antigen gives one band of precipitate and multiple antigens under the proper conditions give multiple bands. The number of bands are equal to the minimum number of antigens present. This last was shown by Oudin and confirmed by us using natural and artificial systems prepared from solutions containing a known number of antigens and a known number of antibodies. The other techniques of Ouchterlony, Elek, of Pope, Bowen, etc., have

[1] Chicago, Illinois, April 8, 1953.

not as yet received the wealth of study in this regard. In this connection the word minimum should be emphasized; the number of bands of precipitate are, so far as present evidence goes, never more than the number of antigen-antibody systems present, but they may be and frequently are less. More will be made of this point later.

Besides the enumeration of the number of antigen-antibody systems, one may use the Oudin and other serum agar techniques for proving the identity or lack of identity of a given antigen in one preparation with an antigen present in another preparation. For this purpose it is necessary to relate a band of precipitate to a given antigen. Later I will briefly discuss the various methods for doing this, and they will be brilliantly exemplified in the work to be presented by Dr. Telfer. One can also obtain some insight into the concentrations of the antigens and antibodies using the Oudin technique although the method has as yet not been developed to the point of providing rigidly quantitative information. It is, I believe, capable of so doing, although the obstacles in the way are formidable at present.

Before proceeding let us see what an Oudin test looks like. The first slide shows an egg albumin anti-egg albumin system with three bands, another preparation of Ea and its antibody showing one band and a horse serum albumin anti-horse serum albumin showing two bands and a bovine serum albumin-anti B.S.A. showing one band.

The tests pictured in the slides are from work done by Dr. Munoz and myself with these various antigen-antibody systems in which we compared the results obtained in the Oudin with the supernatant test for inhomogeneity. In this instance the correlation was perfect; in every case in which we found antigen and antibody in the same supernatant we found more than one band in the Oudin. In instances in which we found only one band, neither antigen nor antibody was found in the same supernate. In subsequent work, I, and probably others, have found systems, which gave supernatant tests indicating homogeneity and yet gave an Oudin with more than one band. The reverse has never been found in my experience. This is undoubtedly a reflection of the fact that the serum agar tests are generally a more sensitive test for inhomogeneity than supernatant analysis. This is true even though the sensitivity of the serum agar techniques for the detection of antigen and antibody is of the same order as that of the precipitin test in liquid media.

While it is generally agreed that antigens of different specificity under the proper conditions can give different bands the question has not infrequently arisen whether antigens of the same specificity but different molecular weight and thus different diffusion coefficients can give rise to different bands. Miss Marion Hyatt, working in my laboratory, has recently completed experiments designed to test this point. The antigens with differing molecular weight were prepared in the following manner. A well washed precipitate of human serum albumin and a horse antibody to human serum albumin was dissolved in antigen excess. By ultracentrifugal analysis the solution showed free antigen and also antigen-antibody complexes of approximately 300,000 *M*, 500,000 *M* and of higher molecular weight. By decreasing or increasing the amount of antigen used to dissolve the precipitate the proportion of higher molecular weight complexes to the low molecular weight complex could be decreased or increased. These complexes were allowed to react as an antigen in the upper layer of an Oudin tube with a rabbit antiserum against horse antitoxin in the agar layer. The rabbit antiserum against horse antitoxin contained antibodies directed toward the horse antibody component of the various complexes. Only one band with a fuzzy leading edge developed, even though both antigen and antiserum concentrations were varied over a wide range and solutions with varied proportions of higher and lower molecular weight complexes were tested. The band was due to the complexes and not to dissociated horse antibody as shown by the fact that the diffusion coefficient of the antigen giving the band was significantly lower than that of simple antibody. Thus, as far as these results go, there is no evidence that antigens of the same specificity but differing molecular weights would give more than one band. This result might be anticipated when it is realized that the more slowly diffusing component is always diffusing in the presence of the faster diffusing component and the more slowly diffusing component is always diffusing in antigen excess.

In examining the tests such as shown in the previous slide, one should note the number and relative position of the bands, the maximum density of the bands, the rate of migration of the

bands and how the precipitate density varies with distance from the leading edge and whether the leading edge is sharp or fuzzy.

The use which can be made of such observations can best be appreciated by first describing in elementary terms, how a band of precipitate develops and migrates. Antigen diffusing down into the gel meets antibody diffusing upward and at the plane of contact the two reactants combine and precipitate. Precipitation continues until no more antibody is available in the very narrow plane of contact, whereupon antigen diffuses further until it comes into contact with fresh antibody and the process repeats.

The advance of the precipitate band into the agar is seen as a resultant of the rate of diffusion of free antigen and the rate of diffusion of antibody. In the picture sketched above, antibody can be looked on as a factor retarding the diffusion of the antigen and thus the movement of the band. The greater the concentration of antibody, the greater is the rate of diffusion of the antibody and the greater the retardation of the movement of the band. Thus the antibody concentration can be so great or the antigen concentration so low that no band will form in the agar. Therefore, in enumerating the number of antigens by means of the Oudin or in testing for homogeneity one should work at the highest possible antigen concentration and at a number of dilutions of antiserum. It is frequently the practice in the literature to state that a given antigen preparation is homogeneous by the Oudin test. From considerations just advanced, it is clear that a declaration of this type should be qualified by a statement of the concentration of the antigen and the concentrations of antiserum used. Without such qualifications, the statement is almost meaningless. In experiments which are designed to test the homogeneity of the antigen preparation and not the homogeneity of the antigen-antibody system it goes almost without saying, that one should also provide evidence that the antiserum used contains antibodies against the possible impurities.

As one dilutes the antiserum, bands frequently appear which were not apparent at the higher concentration. It also is possible that at the lower concentration antibody will not be present in sufficient concentration to give a detectable band, i.e. bands may disappear. If one finds that a given band has a slower migration rate at one concentration of antiserum than another at a lower concentration of the same antiserum, the two bands must be due to different antigens. This follows from the fact that as one reduces the antibody concentration, one reduces the retarding force and the migration rate must increase.

Similarly if a band is present at two different concentrations of the same antiserum and the band at the lower concentration has a greater density than the band at the higher concentration, the two bands must be due to two different antigen-antibody systems. This follows from our elementary picture of the formation of the band. The amount of precipitate which is formed depends on the amount of antibody present at the plane of contact of antigen and antibody, i.e. on the concentration of antibody and on the maximum amount of antigen which can be bound by this amount of antibody without having free antigen present. The ratio of the minimum amount of antigen required to precipitate all the antibody in a unit volume of antiserum to the amount of antibody in that volume we have termed the R′ value. The greater the concentration of antigen, the greater the rate of diffusion of antigen, the faster this minimum amount of antigen will be attained but the maximum amount of precipitate will not be altered. Thus the maximum density of the band is proportional to the antibody concentration but independent of the antigen concentration and this, in fact, has been found experimentally by Oudin.

The observation of the maximum density of the precipitate can give information on several points beside the one mentioned. A very faint band can be stated to be due to an antibody present only in weak concentration. If the faint band is migrating at a relatively slow rate, this means the antigen is also present in relatively low concentration and so on. If one has a preparation which gives only a few bands which differ in maximum density and another preparation with the same antiserum also gives a few bands, those bands which have the same maximum density can tentatively be assumed to arise from the same antigen present in the two preparations. When there are a large number of bands this means of identification becomes less certain.

Behind the leading edge of the precipitate more and more antigen is diffusing and we are in the region of antigen excess. If the antigen-antibody precipitation is inhibited in antigen excess the density of precipitate fades out and the tube actually clears far enough behind the leading

edge. This was actually seen in the Ea tube of the previous slide. The variation of density with distance from the leading edge to the meniscus can be looked upon as a representation, in the round (or at least the cylinder) of the quantitative precipitin curve from equivalence to far antigen excess. The precipitate density is equivalent to the total amount of precipitate while the distance from leading edge to the serum-agar meniscus represents antigen concentration. Unfortunately the concentration of antigen does not increase linearly with distance from the leading edge and the scale for transforming distance along tube to concentration is an unknown function of antigen and antibody concentration. Keeping all this in mind, however, one can frequently make a shrewd guess as to the shape of the antigen excess end of the quantitative precipitin curve given in liquid media from the manner in which precipitate density changes with distance from the meniscus. When one has a band which fades out only a short distance behind the leading edge and the antibody concentration is apparently not excessively low as seen by the density of the precipitate and the antigen concentration not excessively high, one can conclude that one probably has a system which is easily inhibited in antigen excess. Conversely when one has a system in which the precipitate density changes very little with distance from the meniscus and the antigen concentration is not especially low and the antibody concentration not especially high, one could venture the guess that the system is inhibited with difficulty in excess antigen.

The relative positions of the bands can tell one where one can expect to meet the various antigen-antibody systems in the quantitative precipitin curve. The next slide represents the situation I have in mind. In liquid media, on the addition of increasing amounts of antigen to a constant amount of antibody one goes from the equivalence point of one antigen-antibody system to the equivalence point of another system in the order in which the bands representing these systems appear in the Oudin.

Oudin first pointed out that all simple protein antigens gave a sharp leading edge in the Oudin while carbohydrate antigens gave a fuzzy leading edge. This is of course an empirical generalization but from our work and others apparently as far as natural antigens are concerned, a good one. Even in natural systems, however, carbohydrate antigens are not the only cause of a fuzzy edge. Oudin, in unpublished work, has found that the same preparation and concentration of human serum albumin gave a band with a sharp edge with one rabbit antiserum while with another rabbit antiserum a band with a fuzzy edge was obtained. He hypothesized that the latter antiserum had a much greater non-precipitating antibody content than the former. The tentative explanation I wish to offer, for the character of the leading edge, is the following. If there are present antigen and antibody able to react so that free antigen will just appear at only one ratio of antigen to antibody (R′ value) the leading edge will be sharp. If on the other hand different antigen molecules of the same specificity exist but with differing capacity to combine with antibody the leading edge will be fuzzy. This is probably the explanation for the fuzzy leading edge given by carbohydrates. If there are antibody molecules with the same specificity but different combining capacity for antigen, the leading edge will also be fuzzy. This is probably the explanation for the results of Dr. Oudin. Thus, on the basis of the above hypothesis, the character of the leading edge gives us insight as to the homogeneity or lack of homogeneity of the R′ value of the antigen-antibody system.

The use of the Oudin in the qualitative analysis of mixtures is fraught with practical difficulties when one has a mixture containing a large number of antigen-antibody systems. Dr. Telfer will later give a detailed account of the difficulties and the triumphs which can be afforded by the use of the Oudin in such an analysis.

When one has a small number of antigens giving three or four bands and these bands differ in maximum density, one can, as stated previously, frequently make a tentative correlation of the bands given by the two preparations by matching the bands giving the maximum densities. However, even here one should confirm the conclusions so arrived at by one of the methods given below. Oudin has devised three general methods for correlating or identifying bands. If one preparation has fewer antigens than another, one may absorb the antiserum with this preparation and use the absorbed antiserum in the serum agar layer against both antigens. Those bands which are missing or are increased in migration rate must represent antigens present in the preparation used for absorption. If one wishes to draw conclusions as to the

number of such antigens, one should absorb the antiserum at several levels of antigen and should use several concentrations of the absorbed sera in the Oudin test. The second technique employs a specific antiserum to remove one or more antigens from the two antigen preparations. The absorbed antigen is used in the upper layer and those bands which are missing or decreased in migration rate are due to common antigens. The third technique is to dilute one antigen preparation with the other, those bands which are common should give migration rates which fall on a smooth curve when the migration rate is plotted against a fraction of a given antigen. Surgalla, Bergdoll and Dack have recently described an interesting modification of the Oudin technique which in many instances should make the job of identification much easier. They devised a chamber containing a common reservoir of antiserum agar but with multiple compartments for the various antigen preparations. Fusion of a band given by a preparation in one compartment with that given by a preparation in another compartment is direct proof of the identity of the two antigens. Even in this technique, however, care must be exercised in the choice of the antiserum concentration at which one works.

There are a number of ways of measuring the rate of migration of a band. Which one is used depends on the precision required. For work, where one is interested merely in assuring oneself that a given band does move, a simple centimeter rule is sufficient. For work of highest precision, we have devised a technique in which the tubes are kept in a constant temperature water bath and measurements are made with a traveling microscope capable of reading directly to 10 micra and estimating to 1 micron. The distance traversed by a band is a straight line function of the square root of the time as seen in the next slide. Here is plotted the distance of the band from the agar meniscus against the square root of the time. We prefer to express the rate of migration in terms of the slope, or what we call the k value of the straight line. This is so because, as can be seen in this slide and as is frequently the case, the line does not begin at the origin. Mr. Snyder, working in Dr. Munoz's and my laboratory at the University of Illinois, was able to show that the magnitude of the intercept varies with the curvature of the serum-agar meniscus and thus the diameter of the tube; the slope is not affected however by this factor. On first forming, the band conforms to the curvature of the agar meniscus and only after further migration does the leading edge become perfectly horizontal. The magnitude of the intercept, as a first approximation is directly related to the time required to straighten out the leading edge. Any factor tending to increase the time required would increase the value of the intercept and vice versa. This, of course, is not to say that other factors do not influence the magnitude of the intercept but the curvature of the meniscus is certainly a major factor. (In private correspondence, Dr. Telfer independently pointed out the possible role of the shape of the meniscus on the intercept value.)

The rate of migration of the band is a function of the concentration of antigen and antibody and this is one of the properties of the Oudin technique which holds particular promise for the quantitative determination of antigen and antibody. At present, unfortunately, this use of the Oudin technique remains mainly a promise. This is due to the fact that numerous factors besides antigen and antibody concentration affect the migration rate. Some of these factors are the diffusion coefficient of the antigen, R′ value of the antigen, the viscosity contributed by the constituents present in the serum agar layer and the nonspecific augmenting effect of proteins and small ions in the antigen and antibody layer.

It has been shown by Oudin that at sufficiently high concentrations of antibody and sufficiently low concentrations of antigen there is a linear relationship between the log of the antibody concentration and migration rate at constant antigen concentration. There is also a linear relationship between the log of the antigen concentration and migration rate at constant antibody concentration. We have given a theoretical derivation for the form of these logarithmic relationships. In unpublished work using an edestin-anti-edestin chicken serum system, Dr. Munoz and I were able to obtain a straight line by plotting the logarithm of the antiserum concentration against the migration rate. We used the standard curve so obtained and the migration rates given by known dilutions of 'unknown' antiedestin sera at constant antigen concentrations to obtain estimates of the relative antibody content of these various 'unknown' antisera. We found discrepancies up to 50–60% when

the results so obtained were compared to the quantitative precipitin determinations made on the same sera. It is possible that differences in viscosity, which still existed after dilution of the various antisera in the same normal serum were the major factor in such discrepancies.

Mr. Nordstrom, working in Dr. Munoz's and my laboratories at the University of Illinois College of Medicine, obtained errors of the same order of magnitude trying to estimate B.S.A. with a rabbit anti-bovine serum albumin by means of the Oudin. These errors, in certain cases, were related to the nonspecific effects of unrelated proteins added to the B.S.A. It has been pointed out by Oudin that nonspecific substances, proteins, small ions in the antigen layer can increase the migration rate of a band over the rate which is found when these unrelated substances are not present. The magnitude of this effect varies but can lead to a rather large error in the estimation of concentration. These are present difficulties but I am hopeful that in time they can and will be overcome. Even now, if one is cautious in the interpretation of the results obtained and is willing to accept only large differences, one can use the Oudin results in a quantitative fashion as will be shown by Dr. Telfer later.

ESTIMATION OF SERUM PROTEIN FRACTIONS BY IMMUNOCHEMICAL TECHNICS

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IMMUNIZATION of animals with specific serum proteins from heterologous species usually has as a goal the preparation of antisera that will react specifically with the particular serum protein used as the antigen. By means of the quantitative precipitin technique such antisera may then be used to estimate the quantity of the given protein component present in serum or other body fluids. Usually a chemical or physical method exists for estimating specific fractions and results obtained by such methods may be compared with the immunologic determination. Thus iron-binding protein and ceruloplasmin in normal serum can be determined chemically by analyses for iron and copper, respectively. Albumin and gamma globulin can be estimated electrophoretically.

The multiplicity of distinct proteins present in plasma or serum renders difficult the isolation of homogeneous protein preparations. Let us assume that a serum protein used as antigen contains 99% of component *A* and 1% of component *B*. The antiserum obtained by immunization with this serum fraction might contain antibody only to *A* or less likely only to *B*. Usually antibodies would be produced to both components and different antisera would vary in the relative amounts of antibody to *A* and *B*. Now assume the antiserum containing antibodies to *A* and *B* is to be used for the immunologic quantitative estimation of *A* in an unknown serum where the ratio of *A* to *B* is 4 to 1. Upon addition of this serum to the antiserum, a precipitate will form with both *A* and *B* components. The total amount of precipitate will be greater than would have occurred if the antiserum had been specific for *A*. The amount of *A* antigen in the unknown serum in this instance will appear to be excessively high when estimated from the quantitative precipitin curve of the antiserum with the original antigen containing 99% *A* and 1% *B*.

Such difficulties may be avoided in part by careful scrutiny of the interactions of the serum protein fractions used for antigen with the antibody developed to this antigen. Consider briefly the characteristics of a homogeneous antigen-antibody system such as might be illustrated by the precipitin reaction occurring when diphtheria toxin is permitted to react with specific rabbit or human antitoxin (1). When increasing amounts of antigen are added to equal aliquots of antiserum, increasing amounts of precipitate are formed to a maximal point known as the point of equivalence. In the zone of increasing precipitate formation, supernate analyses show the presence only of an excess of antibody. At equivalence, supernate tests show no excess of antigen or of antibody or only a slight excess of antigen. Beyond the zone of equivalence, supernate tests show only an excess of antigen and the antigen-antibody complex becomes increasingly soluble. When the amount of antigen added has approached two to threefold the amount of antigen present at the equivalence point, there is no precipitate. Failure of the precipitate to progressively decrease and to ultimately disappear in the zone of increasing antigen excess is regarded as evidence of inhomogeneity or of cross-reactivity in the system (1–4).

When the ratio of antibody to antigen in the specific precipitate is plotted against antigen added in the zone of antibody excess, a straight line relationship obtains. From theoretical considerations this line may be expressed as

$$Ab/an = 2R - \frac{R^2An}{A}$$

where *Ab* is antibody precipitated, *An* is antigen added, *A* is the maximal amount of antibody in the system and *R* is the ratio of antibody to antigen in the specific precipitate at the zone of equivalence (5). In the diphtheria toxin-antitoxin system, the values for *R* for an antiserum from a given species were remarkably constant from one antiserum to another. Where

the value for R was found to be increased, evidence of inhomogenity of the antigen-antibody system was detected. Here suitable absorption reduced the value for R to the usual range. It is uncertain as to whether a similar constancy for the value for R is to be expected for systems other than that of the precipitin reaction of diphtheria toxin with human, monkey or rabbit antiserum.

An additional criterion for homogeneity is afforded by the Oudin diffusion technique. The presence of more than one band in this diffusion system is evidence of a mixed antigen-antibody system (6, 7).

alpha-1 globulin obtained from human serum. The antiserum contained antibodies to substances additional to the ceruloplasmin. This antiserum was made specific by absorption with serum from a patient with Wilson's disease (hepatolenticular degeneration) where the plasma ceruloplasmin is unusually low. The absorbed antiserum could be used to determine quantitatively the amount of ceruloplasmin in human serum samples.

It is relatively easy to prepare a specific antiserum to crystalline human serum albumin. After calibration, such antisera may be employed for the estimation of albumin in human body fluids. This is impressively demonstrated in the work of

TABLE 1. GAMMA GLOBULIN II-1,2 AND II-3 ANTISERA

Rabbit Antisera to $II_{-1,2}$	Equation for Line	$A_{max.}$ mg protein/ml.	R
X-1	y = 9.8 - 10.5 x	2.09	4.9
X-2	y = 7.4 - 24.1 x	0.56	3.7
X-5	y = 9.0 - 9.6 x	2.12	4.5
X-6	y = 7.0 - 15.8 x	0.78	3.5
W-13	y = 8.8 - 34.2 x	0.56	4.4
Pool-1-I	y = 7.0 - 8.83 x	1.80	3.5
Pool-1-II	y = 11.2 - 7.1 x	4.43	5.6
Pool-4-I	y = 7.1 - 8.9 x	1.42	3.6
Chicken Antisera to $II_{-1,2}$			
A	y = 9.7 - 12.9 x	1.82	4.8
B	y = 7.6 - 8.6 x	1.68	3.8
Rabbit Antisera to II_{-3}			
Pool-I	y = 8.0 - 4.1 x	2.20	3.0

Note the varying values for R, the ratio of antibody to antigen in the specific precipitate at the zone of equivalence. Observe also the differences in the total antibody content (A max.) with the various antisera.

In preparing an antiserum to a specific serum protein, the antigen should be as pure as possible. When gross contamination is present, the amount of antigen used for immunization should be minimal. Multiple individual antisera rather than pooled lots should be tested. The variable antibody response to multiple antigens is illustrated in an experiment in which each of five rabbits was given small amounts of normal human serum. Following immunization, all five antisera showed antibodies to serum albumin. Four had antibodies to gamma globulin, two to iron-binding protein and one to a serum mucoprotein.

In certain instances, absorption of an antiserum containing antibodies directed against an undesirable antigen may make the antiserum specific for the desired antigen. Preferably the protein mixture used for absorption should have a relatively low content of the specific antigen for which the antiserum was prepared. Scheinberg and Gitlin (8) prepared a rabbit antiserum to ceruloplasmin, a copper protein which is an

Gitlin and Janeway (9). From an immunochemical standpoint, the human albumin-antialbumin system fulfills all the criteria for homogeneity outlined above, save for one. In the zone of antigen excess, there is not a progressive decrease in the amount of precipitate. Nonetheless antisera to human albumin are useful for the quantitative estimation of albumin.

Much controversy exists as to the feasibility of estimating the gamma globulin content of human serum by means of calibrated antiserum to human gamma globulin fractions (3, 10–12). It might be mentioned that not only can gamma globulin be separated chemically and electrophoretically into two fractions of differing mobility, designated as gamma-1 and gamma-2 globulins, but that additional components can be obtained by alcohol fractionation or more suitably by electrophoresis convection, a method that has yielded seven distinct gamma globulin fractions in human serum (13).

Several years ago we observed that the values

for the gamma globulin content of human serum obtained by using calibrated antisera (rabbit) to human gamma globulin fractions II-1, 2 and II-3 were excessively high (14). This group of animals had received a total of 30 mg of alum-precipitated antigen. It was considered worthwhile to repeat this study using smaller amounts of antigen in the hope that a more specific antiserum would be obtained. Accordingly a study was made of individual antisera as well as of small pools of antisera of rabbits receiving only 12 mg of alum precipitated II-1, 2 or II-3 globulins by the intravenous route. In addition two chickens were given II-1, 2 antigen dissolved in saline in a total amount of 50 mg/kg of body weight.

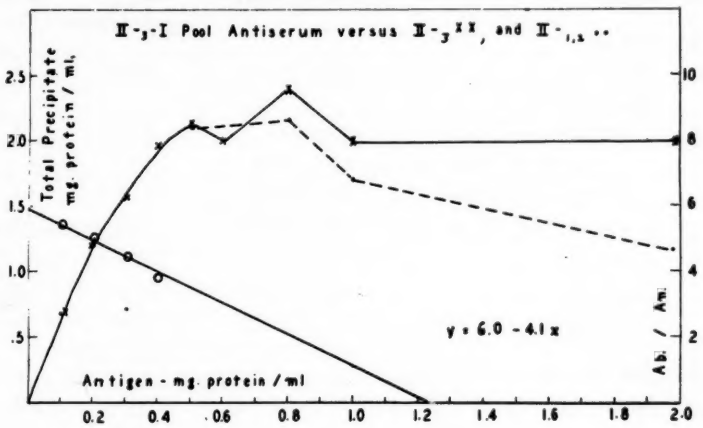

Fig. 1. THE II-1, 2 and II-3 gamma globulins as antigens when added to a rabbit antiserum to II-3 globulin remove identical amounts of precipitate up to 0.6 mg of antigen per ml. Beyond this point (zone of antigen excess), the precipitate with II-1, 2 antigen is less than with II-3 antigen. The line depicts the relationship where the ratio of antibody to antigen in the specific precipitate is plotted against antigen.

Certain of the quantitative characteristics of these antisera are presented in table 1. Instead of the more complicated equation:

$$Ab/An = 2R - \frac{R^2An}{A}$$

mentioned above, we have kept the equation: $y = a - bx$ which describes the line obtained when the ratio of antibody to antigen in the specific precipitate is plotted against increasing amounts of antigen in the zone of antibody excess. For contrast there is included the behavior of one pool of antiserum (pool-1-II) where the animals received a second course of the same amount of antigen. In the animals receiving only 12 mg of antigen, the values for *R* (ratio of antibody to antigen in the specific precipitate at the zone of equivalence) ranged from 3.5–4.9. However in the antiserum obtained by reimmunization, the value for *R* was 5.6. The two chicken antisera differed considerably from each other in the values for *R*. The pooled antiserum to fraction II-3 had a value of 3.0 for *R*.

When antisera to II-1,2 or II-3 gamma globulins are examined by the quantitative precipitin method, there regularly occurs no striking decrease in the amount of precipitate far in the zone of antigen excess. This is illustrated in figure 1.

Up to the zone of equivalence, II-1,2 and II-3 antigen precipitate equal amounts of antibody from rabbit antisera to either antigen. However in the zone of antigen excess, differences between the two antigens often are noted in that the quantity of precipitate to one of these globulin fractions may differ markedly from the amount precipitated by the other, using an antiserum to either globulin fraction (3). Such a response is illustrated in figure 1.

Using chicken antiserum to II-1,2 globulin, Cohn, Deutsch and Wetter (3) found differences in the amount of precipitate formed with equal amounts of II-1,2 or II-3 antigens even in the zone of antibody excess. Using a chicken antiserum to II-1,2, we were unable to detect any difference in the amount of precipitate produced by the two antigens in the zone of antibody excess. The antigen-antibody interaction in our study was made in a solution containing 8% sodium chloride (w./v.).

Each of the gamma globulin antisera listed in table 1 was examined by the Oudin diffusion method. Antisera were overlayed with II-1,2 antigen in concentrations varying from 0.1–18 gm/100 ml and with II-3 antigen in concentrations ranging from 0.1–3.0 gm. In each instance, only one diffusion band was observed. Similarly antisera in agar overlayed with different human sera showed only one band. When normal or abnormal undiluted human serum is treated with 35% saturation with ammonium sulfate, the super-

natant fluid on electrophoretic examination contains no detectable gamma globulin. The various antisera to II-1,2 and to II-3 antigens were overlayed with this supernate and two diffusion bands were observed regularly. This points to inhomogeneity of this antigen-antibody system.

The varying values for R noted in table 1 for different antisera also suggested that the antisera might not be homogeneous. In an effort to find the lowest attainable value for R for this system, the pooled serum from rabbits that had had two courses of immunization with II-1,2 antigen (pool-1-II) was absorbed with small amounts of II-1,2 antigen or with varying quantities of 'fraction II', the protein precipitated from human serum between 35 and 50% saturation with ammonium sulfate. It will be observed that the value for R could be reduced significantly by these absorption procedures (table 2). Absorption of rabbit antiserum to II-3 with the same fraction had no similar effect. However in this instance the value for R in the unabsorbed antiserum was quite low. In an antiserum obtained by pooling the sera of four rabbits immunized with 12 mg of II-1,2 antigen, absorption was effected with serum from a patient with 'agammaglobulinemia' without significantly reducing the value for R. It might be added that rabbit antisera to II-1,2 globulin absorbed with varying amounts of this serum still showed large amounts of precipitate far in the zone of antigen excess as did the unabsorbed antisera. It would appear that the lowest value for R that may be expected for rabbit antisera to II-1,2 or II-3 antigens against the homologous antigens must lie between 3.0 and 3.4.

To further investigate possible differences in individual rabbit antisera to II-1,2 or II-3 globulins, each of the individual rabbit antisera listed in table 1 was used to estimate the amount of gamma globulin in four human sera whose gamma globulin content electrophoretically varied from 10–21% of the total serum protein. The different antisera gave values for gamma globulin in the unknown human sera which were in close agreement with each other and with the electrophoretic values. The II-1,2 pooled antiserum obtained after a second course of immunization gave values for gamma globulin that were excessively high as compared with electrophoresis. After absorption of this antiserum with 'fraction II', this antiserum gave values for gamma globulin that were in close agreement with the electrophoretic values.

A total of 37 immunologic estimations of gamma globulin have been compared with electrophoretic estimations of this fraction using the individual antisera listed in table 1. There was no instance where the gamma globulin values expressed as percentage of the total serum protein differed from each other by more than 3% with the two methods.

There are doubts regarding the reliability of the quantitative precipitin method as a means of estimating gamma globulin content for all abnormal human sera. It has been demonstrated that only 60% of the protein in the gamma-1 globulin fraction reacts immunologically with

TABLE 2. GAMMA GLOBULIN ANTISERA: EFFECT OF ABSORPTION

Antiserum	Equation for Line	$A_{max.}$ mg. protein/ml.	R
II-$_{1,2}$ Pool-II			
Unabsorbed	y = 11.2 − 7.1 x	4.42	5.6
Absorbed with:			
0.36 mg./ml. of II-$_{1,2}$	y = 8.4 − 34 x	0.52	4.2
3.0 mg./ml. of "Fr. II"	y = 8.2 − 88 x	1.91	4.1
5.0 mg./ml. of "Fr. II"	y = 8.5 − 25.0 x	0.72	4.2
3.0 and 1.5 of "Fr. II"	y = 7.2 − 12.5 x	1.03	3.6
II-$_3$ Pool - I			
Unabsorbed	y = 7.0 − 6.8 x	2.20	3.5
Absorbed with 1.0 of "Fr II"	y = 7.7 − 20.2 x	0.70	3.8
II-$_{1,2}$ Pool IV			
Unabsorbed	y = 7.1 − 8.9 x	1.42	3.6
Absorbed with 1.0 of "H.K."	y = 6.9 − 10.8 x	1.10	3.4

Absorption of rabbit antiserum II-1, 2 pool-II with the fraction of human serum precipitating between 35 and 50% ammonium sulfate (fr. II) resulted in a significant decrease in R. Pool IV, a rabbit antiserum to II-1, 2 gamma globulin was absorbed with serum from a child (*H. K.*) with 'agammaglobulinemia'.

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6. OUDIN

antisera to II-1,2 or II-3 gamma globulins. Usually only 1–2% of serum protein is gamma-1 globulin. In instances were gamma-1 globulin is greatly increased, the immunologic method would be unsuited for measuring total gamma globulin. Certain plasma proteins occurring in some cases of multiple myeloma and certain cryoglobulins present in abnormal human serum form precipitates when added to antisera to gamma globulin. Here also the immunologic method for estimating gamma globulin would be unsatisfactory.

Iron-binding protein presents a problem which is quite different from that of human gamma globulin. The iron-binding protein preparations we have used as antigen have contained only 80–92% of the desired antigen. As we have indicated elsewhere (16) by selection of individual rabbit antisera and by suitable absorption methods using human serum, an antiserum may be obtained which appears to react specifically with iron-binding protein.

In our hands mucoprotein from human serum (17, 18) proved to be nonantigenic when injected intravenously as an alum precipitate into rabbits or when injected as a saline solution by the same route into chickens. Yet, as we have indicated above, it was at least slightly antigenic when introduced into rabbits in the form of whole human serum which contained less than 1% mucoprotein.

C-reactive protein appears in the serum of man under conditions of acute tissue injury. It is not present in normal human plasma. Antisera prepared in rabbits to human C-reactive protein react specifically with this substance (19). Acute tissue injury in rabbits also may give rise to serum C-reactive protein. The C-reactive protein of the rabbit serum when introduced into chickens leads to the production of antibodies specific for the rabbit C-reactive protein (20).

The greatest advantage of immunochemical methods for estimation of specific proteins resides in the extreme sensitivity of the method. With this procedure it is possible to measure as little as 10–20 μg of protein in a sample of 1–2 ml. The major drawbacks reside in the labor involved in applying the precipitin technique and far more in the difficulties encountered in preparing specific antisera.

The usefulness of the immunochemical method is illustrated in the examination of a serum from a child with agammaglobulinemia. The child had recurrent infections not unlike those described by Bruton in a similar case (21). Electrophoresis of this serum studied by us showed no distinct gamma globulin peak but did not exclude the possibility that a small quantity of this component might be present. When the serum in varying dilutions was added to calibrated rabbit and chicken antisera to the gamma globulin fraction II-1,2, it was found that the antigen present corresponded to a gamma globulin value of 2.2% for the child's serum. The same 'agammaglobulinemia' serum was used as an antigen in the Oudin diffusion technique. It was layered over a rabbit antiserum to the II-1,2 globulin. There was only one band observed. The rate of diffusion of the band corresponded to one expected for a human serum sample containing 2–3% gamma globulin.

With improving plasma fractionation methods, it should be possible to obtain pure antigenic substances which would provide for preparation of specific antisera to a great number of plasma fractions. Particularly for those plasma fractions occurring in low concentrations, the immunochemical method may provide the most satisfactory means for their quantitative determination. Many difficulties will be encountered in attempts to produce specific antibodies by immunization of animals with impure antigens.

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IMMUNOCHEMICAL ANALYSIS OF EGG WHITE

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CHICKEN egg white (EW) offers an unusually attractive system for immunochemical study. As the consequence of a tremendous effort on the chemistry of this material, the major portion of the proteins as represented by ovalbumin (Ea), conalbumin (Ca), ovomucoid (Ov) and lysozyme (Ly) have been prepared in highly purified form and have been subjected to relatively extensive physical-chemical characterization. The latter three proteins (Ca, Ov and Ly) have the specific *in vitro* activities of iron binding, trypsin inhibition and lytic activity for some bacteria, respectively. These properties and the formation and secretion of the EW proteins in the hen's oviduct and their role in embryological development offer a broad field of biological investigation. The quantitative immunochemical techniques provide an excellent and highly specific protein assay method for the study of problems in this area. The individual immunochemical reactions of the purified components of EW will necessarily be the basis for their assay by such methods in any complex natural system. Thus, the main focus of attention in this work must be initially directed to the preparation of highly purified and characterized proteins in order to provide antigens of the greatest possible immunological purity.

The assay of the egg white system by an independent method for the purpose of immunological reference is best illustrated by the electrophoretic technique. A typical descending pattern of chicken EW in pH 8.6, diethylbarbiturate buffer of ionic strength 0.1, is shown in figure 1. Components recognized in terms of purified entities, namely Ovalbumin (A_1 and A_2), Ovomucoid (Ov), Conalbumin (Ca) and Lysozyme (Ly) are indicated. Only a portion of the area designated Ov is ovomucoid. The component designated F may be a portion of the EW ovomucin. At least two globulins, avidin and several other proteins which make up small amounts of the total system, fail to reveal themselves under the indicated analytical conditions. The amounts of the well-recognized proteins in EW and some of their properties are given in table 1. The nitrogen factors are particularly important in immunochemical assay since the individual proteins of EW vary considerably in nitrogen content. The average percentage nitrogen of the EW proteins is taken as 16. In practice this system is standardized for immunochemical work by determining the total nitrogen of solutions that have been exhaustively dialyzed against 0.15 M NaCl. The salt solution prevents the precipitation of small amounts of globulins. Recourse to this procedure is necessitated by the high solubility of ovomucoid in the usual trichloroacetic acid precipitation technique of determining protein nitrogen.

The preparation of antisera to the various purified EW proteins is best carried out in the rabbit. Although flocculating antibody system as exemplified by horse antiserum is actually more desirable in many respects from an experimental standpoint, it is not usually available. In terms of storage stability, a better defined immunochemical system and related factors, it is often desirable to separate and use the γ-globulin portion of the immune serum. Techniques for this are readily available, the rabbit antibody in particular lending itself nicely for this purpose (1).

Immunochemical Assay of Ovalbumin (EA). Horse serum from animals immunized to three times crystallized Ea was found to give two zones of flocculation when tested against whole EW (2). The second zone of flocculation was later shown to be due to conalbumin Ca, (3). The results of quantitative precipitin reactions of this system with purified Ea and Ca are presented in figure 2. When EW is assayed for Ea by determining the optimal flocculation point for EW and for purified Ea in the Ca absorbed antiserum, a value of 65% is obtained.

The use of rabbit antiserum to six times crystallized Ea provides serum suitable for its assay in egg white. Results of the reaction of Ea and EW with such antiserum are plotted to give figure 3*A*. The ratio of Ea:EW nitrogen

giving identical amounts of specific precipitation in the region of antibody excess shows a value of 61%. The levels of Ea as determined by flocculation and precipitin reaction, respectively, may be compared with the electrophoretic value of 60% reported by Bain and Deutsch (4).

Minor impurities in the six times crystallized Ea are shown by the small amount of specific precipitation with rabbit antibody in the far antigen excess region and the fact that EW shows an increased precipitation in this area. The amount of antibody directed against these components would not appear to invalidate the assay results based on the reactions occurring in the region of antibody excess.

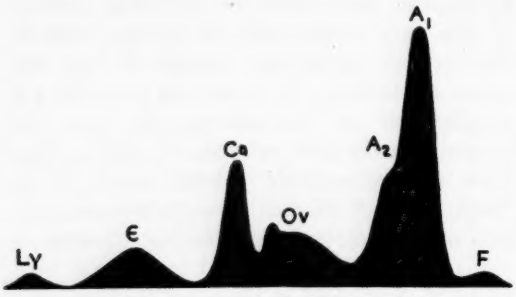

Fig. 1. Descending electrophoretic boundary of egg white in pH 8.6, μ = 0.1 diethylbarbiturate buffer.

Table 1. Properties of purified egg white (EW) proteins

Protein	% N	Mol. Wt.	Isoelectric Point	% Egg White Proteins, Analysis		Antibody N/ Antigen N[2]
				Physicochemical	Immunochemical[1]	
Conalbumin	16.6	87,000	5.8–6.8	16.0(5)	16.0(6)	4.0
Ovalbumin	15.8	44,000	4.6–4.7	60	61 (3)	7.9
Ovomucoid	13.2	27,000	3.9	14.0(9)	13.2(4)	14.6
Lysozyme	18.6	17,200	11.3	3.8(9)	3.7(12)	16.2

[1] Assay with rabbit antibody. [2] Ratios near or at the equivalence point.

Conalbumin (Ca). Initial immunochemical studies of Ca with rabbit antisera showed the presence of a highly antigenic impurity (3). More recent work employed a preparation of Ca in which only small amounts of this impurity were present (5). The reaction of Ca and EW with rabbit anti-Ca serum is shown in figure 3*B*. In the antibody excess region the ratio of Ca:EW nitrogen giving equal amounts of specific precipitate shows that Ca constitutes 16.0% of the EW system. Marshall and Deutsch (6) with another antiserum found a value of 15.9%. The increased amount of the Ca impurity in EW over that in Ca is shown by the greater precipitation given by EW immediately following the antibody excess region and the decreased precipitation in the far antigen excess. The immunochemical assays with rabbit antisera give results which agree well with the electrophoretic value of 16% (4) and thus do not appear to be strongly affected by the impurity.

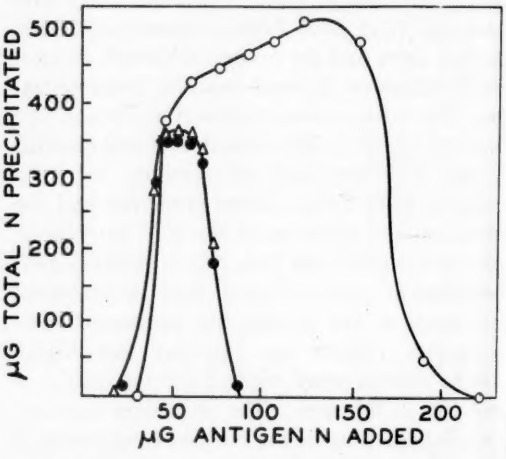

Fig. 2. Reaction of conalbumin -○-○-○-○-, and ovalbumin -△-△-△- with horse serum containing antibody to conalbumin and ovalbumin, -●-●-●-ovalbumin with the conalbumin-absorbed anti-serum.

A somewhat higher level of Ca is obtained by assay with horse anti-serum. The data for the reaction of Ca with horse anti-Ca serum have already been given in figure 2. Determination of the amount of Ca in EW by means of this flocculating system gives a value of 17.8%. Thus, in relation to the results obtained with rabbit antisera, higher values for the Ea and Ca of EW are obtained in the flocculating system.

Ovomucoid (Ov). A detraction in immunochemical studies of this protein is its weak antigenicity. Antiserum of low titer can, however, be obtained from a portion of injected rabbits after prolonged immunization by the adjuvant technique of Freund and Bonanto (7). An example of the reaction of Ov and EW with such antiserum is shown in figure 3*C* (8) from which a value of 9.3% for the amount of Ov

nitrogen in EW may be calculated. Using the nitrogen factors for EW and Ov, respectively, a level of 13.2% Ov in EW is obtained. This is within the relatively wide range of values reported by various workers using electrophoretic (9) and other assay methods (10, 11).

In the case of ovomucoid the results for its immunochemical assay in EW give identical results. The antiserum produced in rabbits to seven times crystallized lysozyme is highly specific and as shown by the data of figure 3*D* can be used to assay EW for its content of this protein. As in the case of Ov the assay values for Ly in the native system obtained from the precipitin curve in both antibody and antigen excess are in excellent agreement. A value of 3.7%

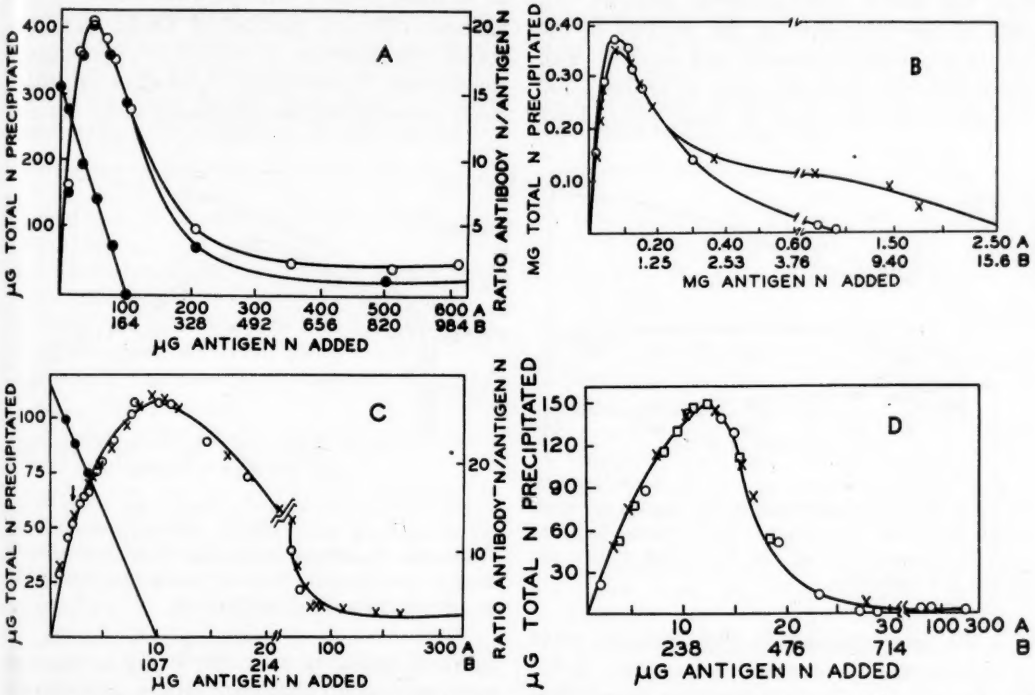

Fig. 3*A*. Reaction of ovalbumin -●-●-●- (*A* abscissa values) and egg white -O-O-O- (*B* abscissa values) with rabbit antisera to 6 times crystallized ovalbumin. *B*. Reaction of conalbumin -X-X-X- (*A* abscissa values) and egg white -O-O-O- (*B* abscissa values) with rabbit anti-conalbumin serum. *C*. Reaction of ovomucoid -X-X-X- (*A* abscissa values) and egg white -O-O-O- (*B* abscissa values) with rabbit anti-ovomucoid serum. *D* Reaction of 7 -O-O-O- and 5 -X-X-X- times crystallized lysozyme (*A* abscissa values) and egg white -□-□-□- (*B* abscissa values) with rabbit γ-globulin to 7 times recrystallized lysozyme.

values in both antibody and antigen excess zones. This precludes the presence of any appreciable amount of impurity in the Ov antigen in contrast to the results found for the Ea and Ca preparations. The prolonged and drastic immunization procedure utilized would likewise have tended to bring out antibody to impurities. The question as to the nature of the slight specific precipitation experienced in the region of large excess of antigen is raised. It is usually suggested to be due to trace impurities.

Lysozyme (Ly). This protein like Ov is only weakly antigenic, and adjuvant techniques of immunization once again give the most favorable for the Ly content of EW results (12) which is close to the electrophoretic assay value at pH 3.92 of 3.8% reported by Longsworth *et al.* (9).

The antibody to antigen molecular ratios of the specific precipitate for most proteins exhibit a decrease of at least 50% from the region of extreme antibody excess to the point of maximum precipitation. In the case of lysozyme this ratio remains fairly constant to near the point of maximum precipitation. Such a result suggests either that lysozyme contains only a limited number of immunologically reactive groups per molecule or that the antigen-antibody complex is restricted because of steric hindrances of a

small antigen reacting with a relatively large antibody molecule. As a consequence of this the lysozyme-rabbit anti-lysozyme system does not follow the Heidelberger and Kendall (13) relation.

Egg White (EW). The immunization of rabbits to whole EW results in the production of large amounts of antibody to components other than the above four discussed proteins (5). Plots of the data showing the reaction of the individual purified proteins and of EW with rabbit anti-EW serum are given in figures 4 and 5, respectively. The first of these plots shows

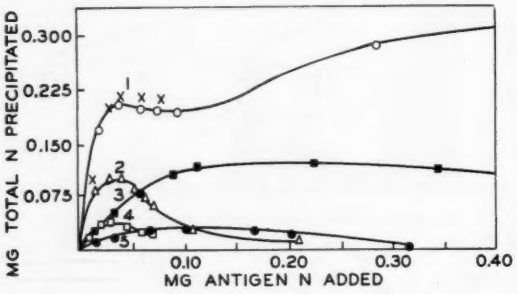

Fig. 4. Reaction of *1* egg white, *2* ovalbumin, *3* conalbumin, *4* ovomucoid and *5* lysozyme with rabbit anti-egg white serum. The crosses *X* represent the summation of the reactions shown by curves 2–5 inclusive.

that the first maximum in the precipitin curve of this system is accountable in terms of Ea, Ca, Ov and Ly. Figure 5, however, reveals that the major antibody production is directed against minor and still unpurified components. One of these, the impurity associated with Ca is responsible for the second maximum of figure 5 which results after the addition of from 0.50–1.0 mg of EW nitrogen per ml of the EW-antiserum employed. As illustrated by the data of *curve 2*, the use of anti-EW serum absorbed with Ca results in disappearance of this maximum. The drop in the amount of precipitation above 4.0 mg EW nitrogen added as illustrated by *curves 2* and *3* is due to the loss of certain labile EW components on aging of this system. The marked antigenicity of the proteins making up only a small amount of the EW system suggests that following their purification further advances in the immunochemistry of the EW proteins will be forthcoming. EW-antisera absorbed with the known major components might well be utilized for assay purposes in preliminary fractionation work directed to the purification of the minor components.

General Remarks. With the exception of Ly, the major EW proteins obey the Heidelberger and Kendall (13) relation up to the point of antigen excess. Rabbit antisera to all of these proteins may be utilized for their immunochemical assay in heterogeneous systems. However, all of these purified proteins when tested by the agar-gel diffusion method of Oudin (14, 15) reveal the presence of two or more antigenic components. In the case of crystalline Ea, three electrophoretic entities designated as A_1, A_2 and

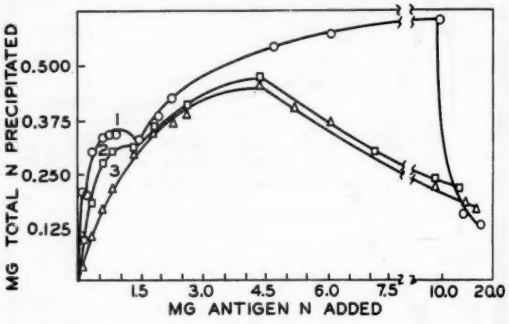

Fig. 5. Reactions of *1* fresh egg white with rabbit anti-egg white serum, aged egg white with ovalbumin absorbed antiserum absorbed with *2* 20 μg of conalbumin N/ml of antiserum and *3* 915 μg conalbumin N/ml antiserum.

A_3 which appear to vary only in the presence of two, one, and no phosphate groups, respectively (16) are present. These appear to be immunologically identical and the nature of the indicated heterogeneity is not known. Possible variations in the immunochemical reactions of the plakalbumins (17) in reference to the parent molecules remain to be explored.

At least a portion of the immunological heterogeneity of Ca resides in a previously discussed impurity. Further work utilizing antisera to Ca of the type employed in our earlier work (3) and crystalline conalbumin as prepared by Warner and Weber (18) may allow for the preparation of a fairly homogeneous antibody to this apparently minor EW protein. Conalbumin is a protein capable of binding two atoms of iron but it is not likely that its immunochemical properties will be influenced by its iron content.

In the case of Ov and Ly the reaction of EW with their corresponding rabbit antibodies gave results in the antigen excess region which sug-

gested that the preparations used contained no impurities. The Oudin Tests, however, indicated the presence of at least two antigenic components. One interpretation of agar-gel diffusion heterogeneity in the face of ability to use antisera to these proteins for their specific assay in heterogeneous system is that the antigenic components of the standardizing (purified) antigens exist in the same ratio as in the native system.

The immunochemical assay for Ea, Ca, Ov and Ly has been so specific as to suggest a distinct advantage over the chemical and physical methods now employed for the determination of these components. The variations in the values for these proteins as determined immunochemically have been slight in comparison with those reported by other methods. Furthermore, their antisera can be used to quantitatively determine extremely small amounts of them in the presence of large amounts of other proteins of related solubility and charge under conditions where present physical and chemical methods fail. Examples of their successful use in systems other than EW have been demonstrated in the assay of Ea and Ca in chicken embryo serum (7) and in determining serum and urine levels of all of the four purified proteins discussed here following their injection into dogs (19).

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ANTIGENIC ANALYSIS OF INSECT BLOOD (*PLATYSAMIA CECROPIA*)

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AMONG the numerous biological phenomena which can profitably be investigated with the techniques of immunology are the growth and differentiation of multicellular organisms. Studies of the time of origin, the site of origin, and the functions of particular antigens during development should contribute to an understanding of both embryonic and postembryonic developmental mechanisms.

In order to exploit fully an antigenic analysis of growth, it is essential that one detect and measure quantitatively individual antigens in the invariably complex mixtures obtained from embryos. Any immunological technique which resolves the total reaction between complex antigen and antibody solutions into its component reactions can thus be useful in an antigenic analysis of growth. For this reason, Oudin's antiserum-agar technique, which Dr. Becker described earlier, has proven to be of value in our investigations of insect metamorphosis. I propose to describe briefly our application of the antiserum-agar technique to an investigation of the role of the blood proteins in the metamorphosis of the Cecropia silkworm, *Platysamia cecropia*.

Metamorphosis in Cecropia begins when the larva stops feeding and spins a cocoon. Within the cocoon, the larva transforms into a pupa, the dormant, overwintering stage of the insect. With the arrival of warm weather in the following spring, the pupa, in turn, transforms into the adult.

The blood of dormant pupae served as the starting point in this investigation because it is readily available at all seasons of the year. There is no clotting mechanism in pupal blood. Erythrocytes are, of course, absent, and there are few leucocytes present. When the leucocytes have been centrifuged down, the blood is ready for analysis. Antisera were obtained from rabbits that had been injected with blood or with saline extracts of larvae, pupae or adult moths.

When pupal blood was reacted in Oudin tubes with these antisera, a maximum of nine bands of antigen-antibody precipitate was visible in any single preparation. If each of these bands can be attributed to a different antigen-antibody reaction, at least nine antigens must be present in the blood of Cecropia pupae. All of these antigens are nondialyzable, heat labile and salted out with ammonium sulfate; thus, they are presumably all proteins. The multiplicity of pupal blood proteins can also be demonstrated electrophoretically. Indeed, electrophoretic patterns of pupal blood are comparable in complexity to those of mammalian serum.

When a system of antigens as complex as Cecropia blood is studied with the Oudin technique, the identification of bands of precipitate requires special attention. It is necessary that one be able to ascertain which of the numerous bands in an antiserum-agar reaction is due to the precipitation of a particular blood antigen. Dr. Becker mentioned the various methods of identification that have been used up to the present time. In our work on Cecropia blood, we have been limited to the use of those methods which do not require the supplementary use of isolated antigen preparations or of antisera which precipitate single antigens.

The problem of identification can be greatly simplified by the selection of appropriate antisera. An antiserum which produces a small number of bands of precipitate when reacted with its homologous antigen solution is preferable to one which produces a large number of bands. A second desirable characteristic of an antiserum is that the bands produced by it each have a density which is readily distinguishable from the density of each of the other bands. For the reasons which Dr. Becker described, when constant antibody concentration is assured, a particular band of precipitate will always have a characteristic density which can serve in its identification as long

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as other bands of similar density are not produced by the same antiserum.

A more reliable method of identification is the 'mutual dilution technique', the nature and application of which may best be described with the aid of examples. Let us consider three antisera, each of which reacted with pupal blood to produce three conspicuous bands of precipitate with characteristic densities. Two of the antisera were obtained from rabbits that had been injected with saline extracts of adult moths. They are designated as adult antisera *a* and *c*. The third, larval antiserum *c*, was obtained from a rabbit that had been injected with a saline extract of mature larvae.

When any one of the three antisera was solidified with agar in glass tubes and overlayered with blood samples from many different pupae, the same pattern of bands appeared in all tubes. With adult antiserum *a*, for instance, the fastest moving band of precipitate always had an intermediate density, the second fastest had the lowest density, and the slowest had the highest density. We have arbitrarily designated these bands of precipitate as bands 1, 2 and 3, and the corresponding pupal blood antigens as antigens 1, 2 and 3, respectively.

These three antigens, which were defined by their reactions with adult antiserum *a*, were also precipitated by adult antiserum *c*, a fact which was readily shown with the aid of the mutual dilution technique. The two antisera were combined in a series of mixtures which varied stepwise from pure adult antiserum *a*, through an equal mixture of the two, to pure adult antiserum *c*. The mixtures were solidified with agar in such a manner that the total antiserum concentration was the same in all tubes. The solidified antisera were each overlayered with an aliquot from a sample of pupal blood, and the *k*-values[2] of the subsequently produced bands of precipitate were determined.

A gradual shift occurred in the *k*-values of bands 1, 2 and 3 from their values in solidified adult antiserum *a*, through the intermediate mixtures to their values in solidified adult antiserum *c* (fig. 1). Each of the bands produced by adult antiserum *c* can therefore be attributed to the precipitation of one of the three antigens that reacted with adult antiserum *a*. In this case, the three bands appeared in the same sequence when the two antisera were overlayered with pupal blood.

Mutual dilution tests also showed that neither antigen 1, 2 nor 3 was precipitated by larval antiserum *c*. The bands of precipitate produced by the reaction of pupal blood with adult antiserum *a*, larval antiserum *c*, and the graded intermediate mixtures of the two showed no gradual transition in *k*-value between the tubes containing the unmixed antisera. Indeed, 6 bands of precipitate

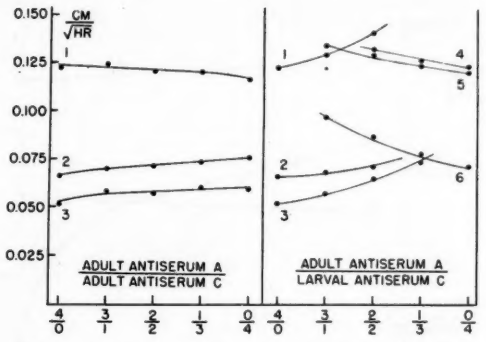

Fig. 1. Comparison by means of mutual dilution tests of bands of precipitate produced in antiserum-agar by different antisera. The *k* values on the *y* axis are those of bands of precipitate produced in reactions of a constant concentration of pupal blood with graded mixtures of antisera (1).

appeared in the tube containing equal parts of the 2 antisera. Thus, we are dealing in this series of tests with six antigen-antibody reactions, and we are justified in designating the bands of precipitate produced by the reaction of pupal blood with larval antiserum *c* as bands 4, 5 and 6.

With the aid of the mutual dilution technique, the bands of precipitate produced by different antisera can thus be identified. The mutual dilution technique can also be helpful in the comparison of the bands of precipitate produced by different antigen solutions. In this case, the antiserum component of the reaction is held constant while the antigen solutions to be compared with each other are mutually diluted. An example of the procedure is the comparison of Cecropia pupal and adult bloods. The 2 blood samples were combined in a series of mixtures which varied stepwise from pure pupal blood, through an equal mixture of the two, to pure adult blood. Each mixture was then reacted in antiserum-agar with adult antiserum *a*.

As increasing amounts of adult blood were

[2] The *k*-value is defined as the distance traveled by the band in centimeters divided by the square root of time in hours.

added to pupal blood in the mutual dilution test, the *k*-values of bands 1, 2 and 3 each changed progressively to the *k*-value of one of the three bands produced by adult blood (fig. 2). We can thus conclude that antigens 1, 2 and 3, whose presence has already been demonstrated in pupal blood, are also present in adult blood. This fact was readily confirmed with absorption tests.

With the aid of these methods of identification, we were able to study the capacity of Cecropia blood at all stages of metamorphosis to produce

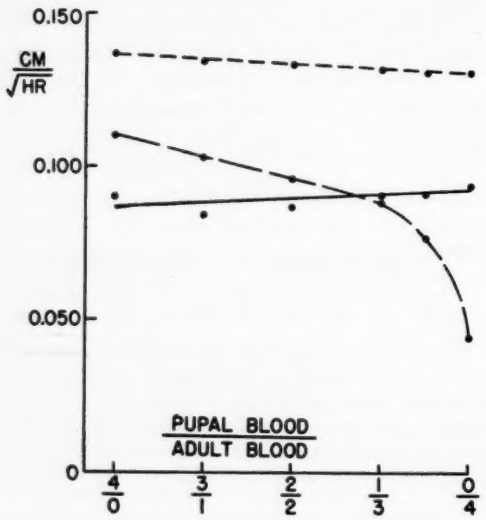

Fig. 2. COMPARISON BY MEANS of mutual dilution tests of bands of precipitate produced in antiserum-agar by reaction of pupal and adult blood with adult antiserum *a*.

seven arbitrarily selected bands of precipitate. The *k*-value of each of the seven bands varied markedly with the stage of metamorphosis from which the blood sample that produced the band was derived. While the pattern of *k*-value change was characteristic for each band of precipitate, there was little uniformity in the behavior of the different bands (1).

A complete description of the various factors which are known to affect the *k*-value of a band of precipitate has already been presented by Dr. Becker. We have analyzed elsewhere the relation of these factors to the system of antigens under consideration (1). From that analysis, we obtained a considerable amount of indirect evidence which indicated that the *k*-value changes observed in the seven bands of precipitate during metamorphosis are attributable to concentration changes of the corresponding blood antigens. If this interpretation is in fact correct, the Ouchterlony technique should prove to be an important tool in studies of the role of the blood proteins in metamorphosis.

A limited number of direct tests of the interpretation of the *k*-value changes in terms of antigen concentration changes during metamorphosis have been provided by two other methods for determining relative antigen concentrations. I first propose to describe the procedure for converting *k*-values to relative antigen concentrations, and then to illustrate the two types of tests to which we have been able to subject the results of this interpretation.

The *k*-value of a particular band of precipitate can, with the aid of a standard curve technique, be used as an index to the relative concentration of the corresponding antigen. In effect, a sample of blood which produces that band with a high *k*-value is diluted with saline until the *k*-value has been decreased to that of the same band of precipitate as produced by a second blood sample. The dilution factor required to accomplish this change is then used as an index to the relative concentrations of the antigen in the two samples of blood.

One test of the validity of this interpretation of *k*-value changes can be readily provided by studies of antigen 7. Antigen 7 is unique among Cecropia's blood antigens because it occurs only in the blood of females. Several of our antisera, after absorption with pupal male blood, retained antibodies capable of precipitating antigen 7 from pupal female blood. In a large number of antiserum-agar tests, run under varying conditions of antiserum dilution and antigen concentration, the reaction appeared as a single band of precipitate, a fact which indicates that the absorbed antiserum precipitated a single antigen from pupal female blood. The results of an analysis of the reaction with the quantitative precipitin technique were also compatible with this conclusion (2). With the quantitative precipitin technique, such an antiserum can be utilized to determine the relative amounts of antigen 7 in different blood samples.

We studied the change in concentration of antigen 7 during the pupal-adult transformation with both the antiserum-agar technique and the quantitative precipitin technique (2). Both methods yielded substantially the same results. Antigen 7 remains at a fairly high concentration

during the first half of adult development, and then falls to a much lower concentration during the last half of development. It appears in this case that the k-value accurately reflects a change in antigen concentration, for the results obtained by the two methods agree both in the direction and in the order of magnitude of the concentration change of antigen 7 during adult development.

As specific antisera are developed for additional blood antigens, it will be possible with the aid of the quantitative precipitin method to test other examples of our interpretation of k-value changes during metamorphosis in terms of antigen concentration changes. In the absence of such antisera, however, we have resorted to an alternative adaptation of the Oudin technique as a supplementary method for the determination of antigen concentrations.

The measure of antigen concentration in the second adaptation of the antiserum-agar technique is the capacity of blood samples to absorb antibodies from an antiserum. The concentration of antibodies remaining in the antiserum after absorption was then estimated by the Oudin technique. To accomplish this end, the supernatant liquid, following absorption, was solidified with agar in glass tubes and overlayered with a sample of pupal blood. The k-value of the band of precipitate in question was then determined. In this case, the k-value was used as a direct index of antibody concentration in the antiserum layer, rather than of antigen concentration in the overlying solution.

In figure 3 the rate of advance of band 7 is plotted as a function of the volume of Cecropia blood that had been used in absorbing the antiserum. Previous absorption with pupal male blood, which lacks antigen 7, had no significant effect on the k-value of band 7. But absorption with pupal female blood resulted in an increase in band 7's k-value, presumably because the antigen 7 present in pupal female blood had precipitated some of the corresponding antibodies from the antiserum. As increasing amounts of blood were used for absorption, the k-value of band 7 continued to increase and, simultaneously, the density of the band diminished until it was no longer visible. We can say that, at that point, all of the antibodies capable of reacting with antigen 7 had been removed from the antiserum.

The relative concentrations of antigen 7 in two different samples of Cecropia blood can be determined by comparing the volumes required of the respective blood samples in order to absorb an arbitrarily selected amount of antibodies from the antiserum. The broken line in figure 3 at k-value, 0.095, represents such an arbitrarily selected point. The respective volumes of the two blood samples required to absorb antibodies from the antiserum to this extent should, when compared with each other, reveal the relative concentrations of antigen 7 in the two samples.

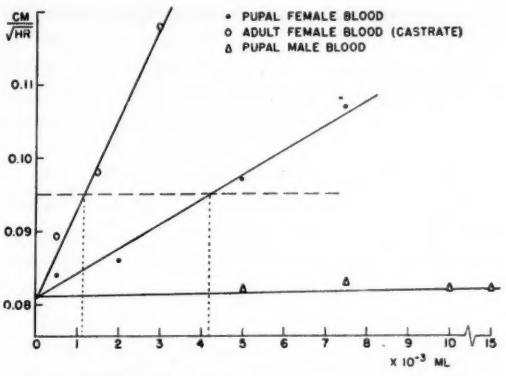

Fig. 3. THE k VALUE of band 7 as produced in antiserum-agar reactions between pupal blood and a standard concentration of adult antiserum d that had been absorbed with various amounts of blood. Type of blood used for absorption is indicated on the figure (1).

We have indicated elsewhere (1) that comparisons of the results obtained by the direct k-value criterion of antigen concentration agree very well with the data obtained by the quantitative absorption method. The fact that two such dissimilar adaptations of the Oudin technique yield mutually consistent results suggests that both methods are valid when applied to the system under consideration.

The reliability of an application of the Oudin technique to quantitative studies of Cecropia's blood antigens is enhanced by two favorable circumstances. One is that the antigen concentration changes which occur during metamorphosis are very large—often on the order of magnitude of several hundred per cent. Secondly, we have been unable to demonstrate in this system the accelerating effects of unrelated proteins on the rate of advance of a band of precipitate. This effect, which was described earlier by Dr. Becker, is one of the main obstacles to a wider application of the Oudin technique to the estimation of antigen concentrations.

Oudin's antiserum-agar technique has provided us with a great deal of information concerning the multiplicity of Cecropia's blood antigens and the complexity of their relation to the morphological changes occurring during metamorphosis. We cannot assert that our present interpretation of the data which we have obtained with the Oudin technique leads to a picture that is accurate in every detail. We are confident, however, that the picture obtained is in general correct. The final test of this assertion will, of course, lie in investigations of the individual blood antigens with the quantitative precipitin technique. For the present, it is fair to say that, in conjunction with other immunological and biochemical techniques, the antiserum-agar technique promises to be an extremely useful tool in studies of the synthesis and functions of individual antigens in developing organisms.

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NATIONAL REGISTER OF SCIENTIFIC AND TECHNICAL PERSONNEL

THE ACCUMULATION of information on scientific and technical personnel, in various degrees of scope and completeness and for a variety of purposes, has been undertaken in the past by both governmental agencies and private groups, including professional scientific societies. Rosters and registers of scientific and technical personnel on a large scale and cutting across several fields of science, as a part of this information gathering process, have been more or less limited to the governmental agencies because of cost considerations and other factors involved. Such agencies have, in turn, initiated rosters and registers in response to emergency conditions, rather than for the primary purpose of developing a long-range, continuing mechanism to study our scientific manpower resources. As short-term, emergency devices previous rosters and registers necessarily have been highly centralized.

The National Science Foundation, in cooperation with several professional scientific societies, has planned a new approach to this pressing national need for reliable and continuing information on the composition, supply, demand and utilization of scientific and technical manpower. This approach recognizes the mutuality of interest between the Government and the representatives of the scientists, the professional scientific societies, in pooling their efforts to achieve a common end.

PREVIOUS ROSTERS AND REGISTERS

The National Roster of Scientific and Technical Personnel was established in July 1940 under the joint sponsorship and direction of the United States Civil Service Commission and the National Resources Planning Board. In April 1942, it was transferred to the War Manpower Commission. Its functions are described in this excerpt from a report of the Director of the Roster to the War Manpower Commission, August 25, 1943:

"The primary function of the Roster is to provide for the most effective utilization of this country's scientifically and professionally trained citizens in the war. Two purposes predominate in the functioning of the Roster, each of equal importance: first, the recruitment of skilled professional and scientific individuals for the Army and Navy and all the war work; second, the best possible utilization and hence conservation of this reservoir of human resources. Through each changing phase in the nation's mobilization, the Roster, in accomplishing its functions has kept foremost its basic purpose of making a proper contact between the right professionally trained man or woman and the right war job."

The most important single activity of the Roster was the development and maintenance of a file of information about persons with specialized training. As of April, 1944, the Roster had in its active files information concerning 472,044 persons who had professional, scientific and/or technical experience.

The Roster was discontinued in 1947. It had served the purposes for which it was intended—the location, registration and placement of persons with specialized skills in a period of mobilization. As organized, it was unwieldy and expensive and not readily adaptable to peacetime needs, but it was used extensively during the war years.

The National Scientific Register, organized in 1950 by the National Security Resources Board, and located in the U. S. Office of Education, was an interim project, pending the assignment of such functions to the prospective National Science Foundation. The Register, after an evaluation of the anticipated functions of the National Science Foundation established two basic policies: *a*) registration should be largely confined to the physical, natural and engineering sciences; *b*) the Register should cooperate fully with the principal professional and scientific societies of the U. S. At the time of transfer of the National Scientific Register to the National Science Foundation on December 31, 1952, about 130,000 scientists had been registered. The National Scientific Register will continue to be used by the Foundation as standby machinery in the event of national emergency and for further studies, pending the establishment of the Foundation's National Register of Scientific and Technical Personnel.

NATIONAL SCIENCE FOUNDATION'S NATIONAL REGISTER OF SCIENTIFIC AND TECHNICAL PERSONNEL

The National Science Foundation Act of 1950 authorizes and directs the Foundation "to maintain a register of scientific and technical personnel and in other ways provide a central clearinghouse for information covering all scientific and technical personnel in the United States, including its Territories and possessions." Soon after the enactment of this act the Foundation reviewed the entire experiences of previous rosters and registers in the United States. Dr. Dael Wolfle, Director of Research, Commission On Human Resources and Advanced Training, as a consultant to the Foundation, was invited to study the problem.

Based upon Dr. Wolfle's study, the following principles were established on which the Foundation plans to develop a register:

1) Decentralization of the register to professional scientific societies.
2) Maintenance of maximum of flexibility, so that the program may be adapted to the changing needs of the societies as well as of the Government and to the changing needs of both in the future.
3) Collection of only essential and justifiable information.

The Foundation has established a 4-point program for the clearinghouse function:

1) To provide standby machinery for identifying and locating persons with special skills in science and technology in the event that in an emergency such machinery becomes essential to national security. The societies will call upon committees to locate people with special qualifications. The register will serve as a tool for these committees. The Foundation will not engage in a placement program short of a national emergency.
2) To assemble statistical information relating to the national supply, training, and utilization of scientific and engineering personnel. Again the register will serve as a tool for special personnel studies.
3) To assemble comparable statistical information on the demand for scientific and engineering personnel.
4) To provide an information center through which information on scientific and engineering manpower resources may be made available to those who seek it.

The register as part of the clearing-house function will serve many purposes. It will be useful as emergency standby machinery and as a basic tool for statistical studies. It will act as a source for scientific biographical dictionaries. Societies may use it in existing placement programs, to assist in the compilation of society directories, to facilitate special surveys of particular interest to the societies, to keep members of the profession abreast of the changing characteristics of the particular scientific field, to assist in career guidance programs, and to determine the composition of the scientific field in order to establish how the society can best serve the profession. In general, registration will be limited to professionally trained scientific and technical personnel having four or more years of experience in a scientific or technical field beyond the baccalaureate degree or its equivalent. Registration will not be limited to society members. Each registrant will be re-canvassed periodically (one to two year intervals) with a brief make-up type questionnaire on the items which are likely to have changed. The following information will be requested of each registrant:

QUESTIONNAIRE

1. Control items—date of information, society membership
2. Name, address
3. Date of birth
4. Sex
5. Citizenship
6. Education—
   - Highest degree
   - Major subject, highest degree
   - Year highest degree granted
   - College or university granting degree
   - Address of college or university
7. Employment (present or last job only)—
   - Name of employer
   - Type of employer
   - Title of position
   - Address of employer
   - Dates of employment
   - Specialty
   - Function
8. Specialties—
   - First specialty, with functions
   - Second specialty, with functions
   - Collateral field, with functions
9. Military status
10. Language proficiency
11. Area specialty
12. Other items desired by the society

Information from questionnaires will be coded on machine punched cards to be retained in the society files, along with the questionnaires. The Foundation will receive a duplicate set of cards from each society.

Thus, the Foundation has planned a relatively simple, flexible, easily maintained register, yet one which will produce the essential information which national security requires.

All the professional scientific and engineering societies have indicated their acceptance of the

principles on which the Foundation has based its register operation. Registers are now being compiled by the following societies with financial assistance from the Foundation:

Federation of American Societies for Experimental Biology
American Institute of Biological Sciences
American Geological Institute
American Veterinary Medical Association
American Mathematical Society (survey only under way at this time)

The following societies have submitted proposals for compilation of a register in their respective fields, or are actively studying the problem to determine how they can best participate in the register program:

American Institute of Physics
American Chemical Society
American Meterological Society
American Psychological Association
Engineers Joint Council

The American Medical Association will collaborate with the Federation of American Societies for Experimental Biology and the American Institute of Biological Sciences in the registration of medical scientific personnel.

On the basis of those societies now actively engaged in establishing these registers and their planned schedule of operations, some 130,000 registrants will be processed by June 30, 1954. One year later the initial registers will be substantially complete. Activities thereafter will be limited to maintenance of the basic register with a continuing endeavor to register a greater percentage of the Nation's professionally qualified scientific and professional personnel. Special procedures will be developed to include the professional societies or organizations of scientists not affiliated with any of the above listed societies.

The activities of the Register are separate and distinct from the current Survey of the Physiological Sciences which is not a registration activity but is a study of the present status of physiology and physiologists. The Register organization and the Survey have consulted on the two projects and have agreed that it is desirable to keep the larger and quite different Survey questionnaire separate from the Register activities.

FEDERATION PLANS

The Federation plans a stepwise procedure in preparing its part of the Register. The first step will be concerned with the membership of the six constituent Societies (about 4000); the second with non-members of the six Societies whose backgrounds and interests place them within these fields (about 8000, including recent Ph.D.'s); the last step will be concerned with 'fringe' individuals. Identification of those to be included in the second step is well under way, and offers no great difficulties. They will include only individuals considered to be professional experimental biologists, with the Ph.D. degree or equivalent.

It was originally planned to send the members of the Federation Societies only a simple postcard questionnaire to bring up-to-date certain of the information items they had supplied for previous registers. Several factors seem to make this plan impracticable, and hence a new but simple questionnaire will be sent to members soon. The information returned will be used for both the NSF Register and for the annual Directory in the December issue of FEDERATION PROCEEDINGS.

While everyone concerned deplores the demands made upon busy people by questionnaires, there is no practicable alternative to use.

MILTON O. LEE
Federation Secretary

REPORT OF SURVEY ACTIVITIES AT THE FEDERATION MEETINGS

THE SURVEY OF THE PHYSIOLOGICAL SCIENCES took a very active part in the recent Chicago Federation meetings.

A 'Wheel of Fortune' showing the distribution of physiologists, research funds, and graduate courses among different physiological specialties highlighted the Survey exhibit. The geographic distribution of physiological Society members, their publication citations and research funds were illustrated on a large, colorful map, and the distribution of physiologists within and outside professional Societies was demonstrated on another wall chart.

Descriptive pamphlets and request blanks soliciting active Survey participation were passed out to exhibit spectators during the five-day meeting. To date, over 40 enthusiastic respondents have returned completed blanks.

Questionnaires designed to determine the effectiveness of relations between the physiological profession and supply, manufacturing, and publishing houses were distributed to 50 commercial exhibitors. An 80% return has stimulated further questionnaire mailings to firms which did not exhibit at the meetings. Complete analysis of all responses is planned by late summer.

Survey participation in the Thursday afternoon Teaching Session included papers on a wide variety of topics by several of our colleagues. Dr. Norma Hajek and Miss Helen Hislop presented a paper on physiological instruction in schools of physical therapy; Dr. Julian L. Solinger discussed the physiological content of elementary biology courses; Dr. E. G. Stanley Baker gave the results of his catalogue study of under-graduate physiology offered by American colleges; and Dr. C. Ladd Prosser outlined the problem of physiological instruction in zoology. Spirited discussion of the problems introduced in the presentations concluded the session.

The generous invitation of the Federation to present a symposium—"A Report on the Survey"—was accepted enthusiastically by the Survey committee. The chairman, Dr. Ralph W. Gerard, delineated the Survey's general objectives in the first presentation. Its social obligations, he urged, are no less important than are its service obligations—to physiology, to biology, to science, to the nation. Availability of Survey results for use by individual, private, and government organizations was also noted by Dr. Gerard in his consideration of the historical background, operating phases, and evaluation of the present and potential status of physiology.

Dr. L. M. N. Bach, Executive Director of the Survey's present definitive phase, discussed operational plans and mentioned the methods to be used in sampling personnel and institutions. The kinds of information already collected and used—personnel and research project rosters, elementary and secondary textbook analysis—were described by Dr. Bach. Evaluating the use of projected studies, he emphasized the imperative need for determining the 'why' of present conditions, rather than merely reaffirming the simple fact that the current situation exists.

In accepting our invitation to present her results, Dr. Anne Roe vividly described her psychological study of the personality patterns of physiologists specifically and biologists in general. In summarizing the personality characteristics manifested by physiologists via the Rorschach and personal interview techniques, she compared them to the results obtained for biologists and scientists in general.

Dr. Orr E. Reynolds, Executive Director during the pilot phase of the Survey, next emphasized the major difficulties encountered in the recruitment and training of physiologists both in undergraduate and graduate programs. Obtaining public recognition of physiology, he concluded, is perhaps one of our most important tasks and weapons against nonsupport of physiological research.

The concurrent Association of American Medical Colleges Survey was explained by Dr. Julius H. Comroe, Jr., the last speaker on the program. He detailed the organization and operational procedures being used by the AAMC in its study of all American schools of medicine and compared them to those used in the Survey of the Physiological Sciences.

L. M. N. BACH
Executive Director
Survey of Physiological Sciences

MILITARY UTILIZATION OF SCIENTIFIC PERSONNEL IN BIOLOGICAL FIELDS

TO ENSURE proper classification (and/or deferment in proper cases) of graduate students and young instructors in the fields of the Federation who are subject to military service, the following information is provided by the Federation office.

1) When an individual receives notice from his local draft board that he is classified I-A, he should immediately submit to the local board a request for reclassification to II-A (occupational deferment) or II-S (student deferment), stating as the reason the fact that he is engaged in essential scientific work. Statements from responsible sources, such as the dean of the medical school or the head of the department or laboratory, should accompany this request. These statements should describe the work in which the individual is engaged, and specify why he is necessary to its completion. If the research is in connection with a defense project, this fact should be brought out.

2) If the request for reclassification is denied by the local draft board, the individual has the right, within ten days, to appeal to the State Appeal Board, through the local board. The material submitted to the local board in support of the original request for reclassification should be forwarded to the State Appeal Board, together with any additional supporting evidence of essential status. The Secretary of the Society with which the individual is affiliated may be requested to furnish a letter in support of the appeal.

3) If the State Appeal Board unanimously confirms the classification of the local draft board, and it is believed that further action is warranted, the State Director of Selective Service should be requested to postpone the individual's induction and call the file in for review.

4) If the State Director denies the appeal, the individual then is subject to immediate induction at the call of his local draft board. He may, however, make further appeal to the Director, National Selective Service Headquarters, 451 Indiana Avenue, Washington 25, D. C. The National Director has the option of reopening the case, at his discretion. If he decides to call in the file for review, induction is automatically postponed until the Director takes action or forwards the case to the National Selective Service Appeal Board for their action.

5) If the individual is inducted by his local draft board, he should *at his initial interview* request assignment in accordance with the Program for Identification, Classification, Assignment and Use of Scientific and Professional Personnel (Regulation SR 615-25-11). For graduate students and instructors in the fields of the Federation, such classification would probably be as Biological Sciences Assistant (MOS 1396; requirements, Master's degree or two years' research) or a related category. The papers furnished by the inductee to his local draft board when making the original claim for deferment should be available for reference at this interview to support the request for classification in accordance with the Program.

Being associated with this Program offers the best opportunity of securing proper utilization of an individual's scientific talents and training as a member of the armed forces, unless there is need for him in a branch of the service which has priority over the scientific program.

6) If, after classification, the individual believes that he has not been properly assigned, the Federation Office will be glad to refer his request for reassignment to the Office of Scientific Personnel of the National Research Council, which is concerned with the proper utilization of scientific personnel, and is prepared to render assistance in every way practicable. Full details of the case, with supporting correspondence, should be addressed to Dr. M. O. Lee, Federation Secretary, 2101 Constitution Avenue, Washington 25, D. C

PREPARATION OF SCIENTIFIC EXHIBITS

SCIENTIFIC INVESTIGATORS have learned that exhibits are an excellent way of presenting the results of their research. The scientific sections of medical and scientific meetings currently draw a great deal of attention. It was observed at the recent American Medical Association convention in New York City that many doctors spent as much as two or three days studying the scientific exhibits. This method of presentation enables the investigator to report his findings and, at the same time, to dispense reprints or booklets discussing his observations and conclusions. The free discussion possible between viewer and demonstrator also promotes a better understanding of the material.

There are many advantages to the presentation of findings in the form of an exhibit.

a) Greater retention and better impressions are possible through a more visual presentation. Certainly no mere word sequence can be as vivid or as effective as a well planned chart or drawing.

b) The attendance at the American Medical Association meetings range, for example, from 12,000–17,000, whereas the majority of scientific journals have a readership range from 2,000–20,000 with an average readership of approximately 8,000. In addition, groups all over the country can see a single exhibit, for there are no restrictions on the number of conventions at which one can present the same material. The average attendance for paper presentations at such a meeting is usually below 100, whereas the attendance at a single exhibit, judging from the number of booklets or reprints distributed, ranges from 4,000–10,000. A report or paper, in contrast, can be published only once.

c) An exhibit can be used to complement the presentation of a paper at a particular scientific conclave. Many times the subject is so involved that just listening will not suffice. In addition, the pertinent facts and salient features of a project can be driven home pleasantly to the viewer with a minimum receptive effort on his part.

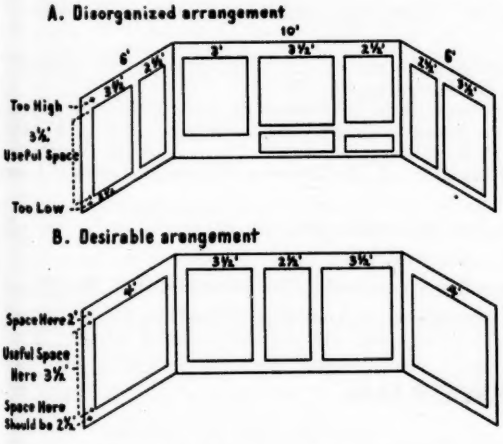
In preparing an exhibit, the exhibitor must first decide what pertinent facts he wants to present in the space available. If he is going to show a technique, then he must be sure to include the subject of the technique, the apparatus, the differences between old and new techniques, results, summary and conclusions. The outstanding features must not be crowded or diminished in importance by inclusion of less significant data. In an exhibit one must remember that one idea, presented singly and clearly, is preferable to a disjointed presentation of three or four poorly defined ideas. An optimum number of illustrations and tabular material will be more appealing and effective than even the best written paper. Also, an exhibit can be made attractive through the judicious use of a second color without resorting to gaudiness. When results of medication are to be presented, it is necessary for the investigator to prepare his data most carefully, for an exhibit loaded with text and numbers is practically of no value.

Once the data has been chosen, the exhibitor must briefly lay it out in the space allotted. For example, if he has two 6′ wings and a 10′ back wall, he can tentatively arrange the exhibit as shown in the figure. It must be pointed out that the fewer the number of posters, the less jumbled the exhibit appears to be. The exhibit with many 1′ squares looks like a checkerboard and the eye jumps from poster to poster, making it difficult to focus on the idea. Also, a small poster does not permit the use of large enough type. The final size of the individual letter should be blown up to at least ¾″ for good legibility. Anything smaller is difficult to read at a distance of 6 feet. Titles, of course, should be as large as possible, within good taste.

Once a preliminary sketch such as the one shown (p. 745) has been made, the exhibitor can then drop his material into the slots they best fit. With three sections as shown, one can have three divisions. The one on the left for *method and material*, the one in the center for *results*, the one on the right for *summary and conclusions*. Actually, each section can be thought of as a group of paragraphs in a paper; the individual posters as the individual paragraphs, and the material on the poster as the individual sentences in the paragraph. Some exhibits do not have side panels and the entire space is along the backdrop. Nevertheless, material should be divided into sections for clarity.

One of the advantages of a scientific exhibit is that the investigator, particularly the pharmacologist and the physiologist affiliated with a hospital or university, can have available to him many individuals who can, if proper interest is created, help him prepare his exhibit. Many

hospitals have photographic and art departments which can aid him in photographing material and in obtaining specimens. The simplest method of presenting an exhibit is to varitype the material in a pleasant and clear-cut arrangement, to photograph and blow it up, and then to mount it on a board. Color can be added by hand. Many technicians will help and cooperate if they have worked on the project and obtained the data in their laboratories. Students can be encouraged to cooperate and contribute their artistic talents. One way to encourage students to participate is to have them present data in the form of a scientific exhibit as part of their class work, rather than in the more tedious report form. They will have to do the same research work either way. They can be rewarded by having



their names on the exhibit or be given some other prize such as a worthwhile text, or needed apparatus. The cost of an exhibit can be maintained at a minimum by using all facilities available in institutions.[1]

It was mentioned earlier that booklets and reprints can be used to advantage when presented with an exhibit. The man who attends a convention usually does so for several reasons. He wants to hear lectures, to see both scientific and technical exhibits and to have a good time. His day is busy and crowded. He talks to and sees many individuals who constantly present new material of interest to him. Because a human being's mind cannot possibly retain this hodge-podge of information, it has been found that a booklet is helpful to remember material of value and interest. What should the booklet be? Most investigators who have prepared and published a journal article can have available reprints of that article. For those who present the exhibit primarily as a means of reporting their data, it is possible to prepare a booklet which reproduces the exhibit in its entirety. In addition, material amplifying and explaining the ideas behind the research can be included. These booklets need not be elaborate and can be prepared quickly and simply. Mimeography may be sufficient. This permits reproduction of sample charts and drawings. If halftones are to be reproduced, it is necessary to resort to photo-offset.

Both clinicians and non-clinicians have shied away from preparing scientific exhibits because they felt it too cumbersome, too expensive and too time consuming. We hope this paper will encourage many of them, for we believe this to be an ideal way of presenting scientific and research data. A simple but attractive exhibit can be prepared at very little cost. If the material and data are readily available, it takes a minimum amount of work to organize it in an orderly fashion. All that remains is the photographing and blowing up of copy. There are many talented individuals affiliated with medical and research institutions who would willingly cooperate in the preparation of such an exhibit. The enlistment of grateful patients in preparing the exhibit often serves a twofold purpose: psychotherapeutic value to the patient and practical assistance to the physician.

With an exhibit the exhibitor may be present to answer questions and to take an active part in discussion which may increase future research possibilities or which may inspire others to enter the same research fields. A paper published in a journal cannot answer any questions. It is impersonal and hasn't any human appeal. A good exhibit can reflect the personality of the man behind the work. A simple, well planned exhibit can reach a good audience from many parts of the country. It can be remembered for a long time through the well planned use of an adequate booklet or reprint. Truly, and rightly so, the scientific exhibit section of many meetings can be the most popular, helpful and informative.

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I wish to extend to Paul de Haen and Julien A. Garbat my appreciation for their assistance in preparing this paper.

[1] Companies such as the Independent Blueprint, Inc., in New York City, can add color by various photographic techniques, or add interest by printing material on backgrounds other than paper.

AAAS BOSTON MEETING

DECEMBER 26–31, 1953

The 120th Meeting of the American Association for the Advancement of Science will include sessions of all 18 AAAS sections and of some 50 participating organizations. Programs of particular interest to members of the Federation are:

I. **British AAS.** An address by A. V. Hill, past president of the British Association, **Dec. 27.**

II. **AAAS.** Presidential address by Detlev W. Bronk, retiring president, **Dec. 28.**

III. **AAAS Section B-Physics.** Symposium on Biophysics, arranged by Richard S. Bear, **Dec. 29.**

IV. **AAAS Section C-Chemistry.** Six-session symposium on nutrition and food technology, arranged by Robert S. Harris, Charles N. Frey, Harold J. Humm, P. W. Zimmerman, and B. E. Proctor, **Dec. 28–30.**

V. **AAAS Section F-Zoological Sciences.** Vice presidential address of Paul Weiss after zoologists' dinner, **Dec. 29.**

VI. **AAAS Section I-Psychology.** Invited papers on Brain Function, Sensory Processes, **Dec. 29, 30,** respectively.

VII. **AAAS Section M-Engineering.** Symposium on "Electronic and Other Aids to the Blind," arranged by Eugene F. Murphy.

VIII. **AAAS Subsection Nm-Medicine.** Four-session symposium on "Antimetabolites and Cancer," arranged by Cornelius P. Rhoads and Allan D. Bass, **Dec. 28, 29.**
Participants include: D. W. Wooley, William Shive or E. M. Lansford, Jr., Arnold D. Welch, John R. Totter, Howard E. Skipper, George Mandel, George H. Hitchings, Sidney Farber, Bernard D. Davis, George B. Brown, Frederick S. Phillips, Donald W. Visser, Joseph Burchenal, Roy Hertz, Sidney Weinhouse, Robert E. Parks, Jr., J. B. Thiersch, David R. Ginder, Louis Nickell, and Cornelius P. Rhoads.

IX. **AAAS Subsection Nd-Dentistry.** Three sessions on dental research, arranged by Howard R. Marjerison, **Dec. 29.**

X. **AAAS Subsection Np-Pharmacy.** Four sessions, cosponsored by six societies, **Dec. 28, 29.**

XI. **AAAS Section P-Industrial Science.** Joint program with American Industrial Hygiene Association, **Dec. 28.**

SOCIETAL PROGRAMS

Alpha Epsilon Delta. Annual luncheon, **Dec. 29.**

American Association of Hospital Consultants. A symposium, "The Research Function of the Hospital," arranged by E. M. Bluestone.

American Institute of Nutrition. The AIN, just affiliated with the AAAS, will co-sponsor Section C's symposium.

American Physiological Society. A symposium arranged by L. M. N. Bach.

American Psychiatric Association. A symposium arranged by Jacob E. Finesinger.

American Society of Human Genetics. During the period, **Dec. 28–30,** there will be three sessions of contributed papers and two symposia: "Human Genetics and Medical Education" and "Genetic Factors Affecting Intelligence."

American Society of Naturalists. Symposium on "Cytogenetic Effects of Radiation from Nuclear Detonations," arranged by Alexander Hollaender.

American Society of Zoologists. During the four days, **Dec. 27–30,** there will be sessions of papers on cytology, embryology, endocrinology, experimental biology, parasitology, and cellular and general physiology, two symposia, and demonstrations.

The AAAS Annual Exposition of Science and Industry and the AAAS Science Theatre, with recent foreign and domestic films, will be prominent features of the Meeting. **Coupons for hotel reservation and advance registration will be found in SCIENCE and THE SCIENTIFIC MONTHLY.**